SCIENTIST REPORT

DEVELOPING THE SECOND GENERATION OF IMPROVISED EXPLOSIVE DEVICE DETECTOR DOG

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14. ABSTRACT

A number of approaches have been used to detect the presence of Improvised Explosive Devices before they detonate. One option taken by the U.S. Marine Corps has been the deployment of improvised explosive device detection dogs (IDDs). The IDD program relies on unique off-leash dog and handler teams, and uses hunt-bloodline, field-trial trained Labrador Retrievers exclusively. The research described in this report represents an important multidisciplinary effort to better characterize stress responses, cognition, and olfaction in Labrador Retrievers, drawing on the expertise of North Carolina State University (NCSU) College of Veterinary Medicine (CVM) scientists with research and clinical backgrounds in veterinary behavior, nasal toxicology, laboratory animal medicine, olfaction, and behavioral sciences. Research was performed in controlled laboratory experiments and field studies and consisted of ten distinct research phases Phase I. Evaluation of the USMC Emotional Reactivity Test Phase II. Development of an Open Field Anxiety Test Phase III. Object Discrimination Phase IV. Delayed Non Match to Position (DNMP) Phase V. Olfactory Discrimination Phase VI. Cognitive Bias Phase VII. Application of Remote Telemetry to a Novel Open Field Test of Olfaction Phase VIII. The Role of Olfactory Priming on the Detection of C4 Phase IX. Soil Depth and its Impact on Odor Detection in Dogs Phase X. Pilot Studies Examining Proton Pump Inhibitor Effects on Canine Olfaction This work resulted in an improved understanding of the strengths and weaknesses of the Emotional Reactivity Test (ERT), a primary tool used by the USMC to select candidate IDDs. Our research identified areas where the ERT was a highly effective test instrument, but also showed that the ERT was less effective as a tool for screening dogs for cognitive or olfactory abilities. The short-term cognitive bias test and canine olfaction assessment test, developed as part of this research, showed promise as practical screening tools for these functions. Our open field model produced a measurable anxiety/stress behavioral response in dogs and provided validation for the NCSU ERT test. Extensive cognitive testing clearly demonstrated individual difference in learning rates, and suggested that dogs with lower emotional resilience and/or an anxiety phenotype might have more difficulty learning new tasks under stressful conditions. Olfactory discrimination studies demonstrated limited ability of dogs to generalize between chemically related samples, and that commonly used screening tests were unable to predict olfactory performance in these dogs.

15. SUBJECT TERMS

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ABSTRACT

Improvised explosive devices (IEDs) remain a significant worldwide threat to civilian and military personnel. In Afghanistan, IED attacks have accounted for a large proportion of the casualties seen in U.S. and Coalition forces. A number of approaches have been used to detect the presence of IEDs before they detonate. One option taken by the U.S. Marine Corps has been the deployment of improvised explosive device detection dogs (IDDs). The IDD program relies on unique off- leash dog-handler teams, and uses young Labrador Retrievers exclusively.

The research described in this report represents an important multidisciplinary effort to better characterize stress responses, cognition, and olfaction in Labrador Retrievers, drawing on the expertise of North Carolina State University (NCSU) College of Veterinary Medicine (CVM) scientists with research and clinical backgrounds in veterinary behavior, nasal toxicology, laboratory animal medicine, olfaction, and behavioral sciences. K2 Solution's role as a prime contractor for the procurement and training of candidate IDDs brought additional expertise to the project. Research was performed in two broad domains (controlled laboratory experiments conducted at NCSU and field studies performed at K2) and consisted of ten distinct research phases:

- Phase I. Emotional Reactivity Test
- Phase II. Development of an Open Field Anxiety Test
- Phase III. Object Discrimination
- Phase IV. Delayed Non Match to Position (DNMP)
- Phase V. Olfactory Discrimination
- Phase VI. Cognitive Bias
- Phase VII. Application of Remote Telemetry to a Novel Open Field Test of Olfaction
- Phase VIII. The Role of Olfactory Priming on the Detection of C4
- Phase IX. Soil Depth and its Impact on Odor Detection in Dogs
- Phase X. Pilot Studies Examining Proton Pump Inhibitor Effects on Canine Olfaction

A unique feature of this project has been the ability to longitudinally follow a single cohort of Labrador Retrievers from an initial assessment of emotional resilience and stress responses, through a battery of cognitive function tests, and finally through an assessment of olfaction. This work resulted in an improved understanding of the strengths and weaknesses of the Emotional Reactivity Test (ERT), a primary tool used by the USMC to select candidate IDDs. Our research identified areas where the ERT was a highly effective test instrument, but also showed that the ERT was less effective as a tool for screening dogs for cognitive or olfactory abilities. The short-term cognitive bias test and canine olfaction assessment test, developed as part of this research, showed promise as practical screening tools for these functions. Our open field model produced a measurable anxiety/stress behavioral response in dogs and provided validation for the NCSU ERT test. This model could be used in future experiments to examine mitigation strategies in candidate IDDs. Extensive cognitive testing clearly demonstrated individual difference in learning rates, and suggested that dogs with lower emotional resilience and/or an anxiety phenotype might have more difficulty learning new tasks under stressful conditions. Olfactory discrimination studies demonstrated limited ability of dogs to generalize between chemically related samples, and that commonly used screening tests were unable to predict olfactory performance in these dogs.

INTRODUCTION

Improvised explosive devices, also known as IEDs, roadside bombs, and suicide car bombs, have caused the majority of American combat casualties in Iraq and Afghanistan (Wilson, 2007). Many counter-IED measures exist and the U.S. Department of Defense (DOD) has established the Joint IED Defeat Organization (JIEDDO) to investigate countermeasures along with various national laboratories, the Department of Energy, contractors, and academia. One counter measure that the U.S. Marine Corps (USMC) uses is Improvised Explosive Device Detector Dogs (IDDs). The USMC IDDs are adult Labrador Retrievers that typically have a background in field trials. One civilian contracted company, K2 Solutions Inc. (K2), trains those canines accepted into the detection program to identify explosives. After the dogs are trained, they are paired with a Marine handler and sent overseas to help locate explosives used by enemy fighters.

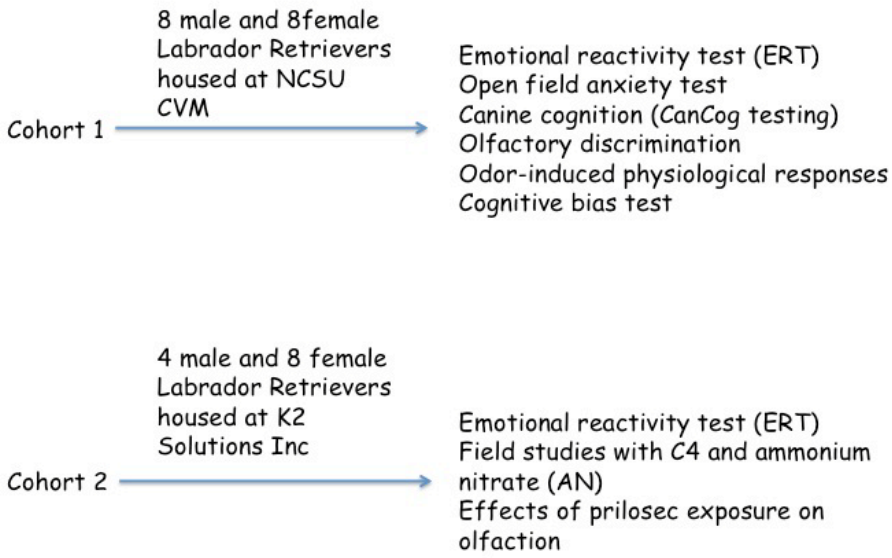
PURPOSE

The purpose of this project is to better understand the odor-detecting abilities of dogs (*Canis familiaris*) in order to optimize their effectiveness in the field. In addition, we sought to enhance the well being and functionality of these working Labrador Retrievers, by developing methods to assess the effects of stress on canine performance. Our research evaluated: (a) the current selection protocol for emotional resilience, (b) stress responses using behavioral and physiological methodologies, (c) cognitive function in dogs, and (d) olfactory function in dogs using laboratory and field-based experiments.

STUDY DESIGN OVERVIEW

Most experiments involved the use of two cohorts of dogs housed at either the North Carolina State University (NCSU) College of Veterinary Medicine (CVM) or K2 (Figure 1). Demographic information about the two cohorts is presented in Table 1. The dog's prior training at the K2 facility is presented in Table 2. Method development occasionally used privately owned dogs to allow the experimental test subjects to remain naïve to the test until the time of study. Data from these pilot efforts are not reported in this document.

Figure 1. Cohorts used in NCSU experiments and overview of tests performed on each group (see main body for additional details).



Research conducted with Cohort 1 followed this overall sequence: animal quarantine, application of an emotional reactivity test (ERT), open field (OF) test for anxiety, and operant conditioning training using the canine version of the General Test Apparatus (CanCog). The CanCog system was used for visual object discrimination, visual object reversal, visual delayed nonmatch to position (DNMP), olfactory discrimination (vanillin), and olfactory discrimination (ammonium nitrate [AN]). Dogs progressed through this phase of training at different rates. Because some dogs in Cohort 1 entered the project with prior olfactory training (Table 2) we used vanillin as the initial test odorant.

Our primary field research conducted with Cohort 2 followed this overall sequence: animal training to odor (AN and C4), confirmation of odor-cued covers behavior (surface trials), cover behavior on simulated buried AN, timed AN and C4 surface trials, and timed AN buried odor trials. Once this baseline was established dogs were put on a therapeutic dose of a gastric acid (proton pump) inhibitor (Prilosec) and retested on their ability to detect surface AN and C4.

Additional experiments were conducted with both cohorts and are described in this report. Certain studies in our initial research project proposal (e.g., evaluation of imprinting methods, evaluation of naïve (odor) dogs as they progressed through the training program) were not attempted since the dog procurement process changed once the study was underway. Also, the group size available for field studies was reduced by 50% from our original design. The changes to the proposed studies were made with consultation with the study sponsor (K2) and members of the funding agency (Office of Naval Research [ONR]).

ORGANIZATION OF THE REPORT

This document is divided into three main sections: (I) general methods and approaches common to both cohorts of dogs; (II) studies performed at NCSU CVM, and (III) field studies performed at K2. Background, methods, results, and conclusions for laboratory (section II) and field (Section III) studies are contained in each section. In some cases (e.g., conduct of the emotional reactivity test), data collected on cohort 2 was used to further confirm the utility of test instruments developed at NCSU. In these cases, we present this data in the section devoted to NCSU laboratory studies.

KEY STUDY PERSONNEL

David C. Dorman, DVM, PhD, DABVT, DABT: Principal Investigator. Oversight for all phases of the research project.
Barbara L. Sherman, MS, PhD, DVM, DACVB: Co-Investigator. Directed the evaluation of emotional reactivity and stress responses in dogs.
Margaret E. Gruen, DVM, MVPH, DACVB: Co-Investigator. Assisted with the evaluation of emotional reactivity and stress responses in dogs.
Richard E. Fish, DVM, PhD, DACLAM: Co-Investigator. Assisted with veterinary care and telemetry data collection and analysis.
Melanie L. Foster, BS, DABT: Co-Investigator/Research Associate. Involved in all facets of the behavioral work involving dogs. Telemetry assessments.
Beth Case, BS, MS: Research Assistant. Assisted with the evaluation of emotional reactivity and stress responses in dogs.
Lucia Lazarowski BA, MA: Research Specialist. Performed all operant training including visual and olfactory discrimination tests.
Amanda Jeffries, BS: Summer Intern. Cognitive bias test.
Matt Clark, BS: Summer Intern. K2 field trials
Heather Waterman, BS: Summer Intern. Telemetry and olfaction laboratory studies.

SECTION I: GENERAL METHODS

MATERIALS AND METHODS

Animals

Young (~1-3 year old), male and female, field-trial-bred Labrador retrievers (Tables 1 and 2) were acquired by K2 and were initially sent to the K2 canine training facility (Southern Pines, NC) for processing and quarantine. Dogs arrived from field-trial-breeding kennels throughout the United States.

- *Animal welfare oversight:* The experimental protocol was reviewed and approved by the NCSU Institutional Animal Care and Use Committee (IACUC) and the DoD US Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO). NCSU IACUC approval occurred on July 13, 2011 (IACUC # 11-093). ACURO approval (NRD 734) was provided August 3, 2011.
- Appendix 1 includes the original NCSU animal protocol and amendments as well as the approved original DOD animal use protocol.
- *NCSU animal facility:* Research conducted at NCSU was performed within the CVM's

Laboratory Animal Resources (LAR) unit. The facility is inspected semiannually by the NCSU IACUC, and the CVM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

- *K2 canine facility:* The K2 Training Center is designed, equipped, and operated to comply with Title 9, Code of Federal Regulations, parts 1-3 and with DoD Directive 3216.01 to guarantee the humane, safe and necessary use of canines. An NCSU IACUC inspection of the K2 kennels was led by the NCSU Attending Veterinarian (Dr. Steve Dempsey) and was held November 7, 2011.
- *Odor training*: Some, but not all, dogs were trained by K2 staff for the detection of AN, C4, and other explosive training aids. Odor training involved two phases: (a) directional control and general obedience training; and (b) odor training using either odor wall and/or open field imprinting methods. Upon detection of the odor the dogs were trained to signal the presence of an odor by sitting or lying down (lying down is also called "cover"). Upon successful demonstration of this behavior the dogs were rewarded by their trainers through play (e.g., retrieving a tennis ball or Kong toy).

Animal Husbandry, Cohort 1

- Animal housing and the majority of the behavioral assessments were performed in a collection of out-buildings referred to as Dog Facilities 1, 2 and 3 (DF1, DF2, and DF3). Dogs were housed in DF3, an environmentally-controlled, cinder block building containing 18, 5 x 8' solid-floor pens, each with a raised resting surface. Temperature set point was 72 F, and relative humidity kept between 30-70%. Temperature and humidity were recorded daily by animal care technicians (ACT).
- Dogs were provided continuous access to water in stainless steel buckets or bowls, and fed Iams Mini Chunks twice a day in an amount to maintain appropriate body condition. (Several dogs were prescribed diet changes for medical reasons; see below). Large stainless steel balls ("Portion Pacer" balls) were added to the food bowls to slow eating.
- Dog runs were cleaned twice daily, and sanitized weekly with disinfectant (Virkon).
- Dogs were turned out on a concrete slab daily during cleaning for exercise, and also were hand-walked twice a week for 15 minutes (in addition to walking to DF2 and DF3 for testing). All dogs received hard rubber toys in their runs during the day.
- Routine grooming (minimally bathing and trimming toe nails) was provided monthly, and more often as needed.
- Each dog in cohort 1 had an implanted microchip that was checked on arrival at NCSU. Each pen had a cage card with the dog's identification, and dogs wore a name collar whenever removed from the facility.

Veterinary care

Animals housed at the K2 facility were under the daily supervision of that organization's veterinary technicians and on-call or on-site veterinarian. Medical records for each dog were maintained at that facility.

Each animal in cohort 1 received a physical examination upon arrival (29 Nov 2011) at the NCSU facility. Dogs were continued on monthly heartworm preventative, initially oral ivermectin, changed subsequently to topical selamectin (Revolution, 240 mg). All dogs were observed daily by trained animal care staff for health or behavioral abnormalities, which were reported to LAR Veterinary Services. Dogs with reported abnormalities were examined on the day reported by a veterinary technician, and veterinarian as indicated. A veterinarian was

available at all times, including after-hours/holiday/weekend, and reviewed all Veterinary Services reports.

Animal Health Summary, Cohort 1

Dogs were healthy on arrival, and remained in generally excellent health while at NCSU. Despite some clinical signs reported commonly, and specific clinical concerns in a few dogs (discussed below), all the dogs remained bright, alert, responsive, and active, with excellent appetites.

General comments:

- It was relatively challenging to maintain ideal body condition in these dogs, in part because they were not kept on a routine exercise program and, therefore, subject to more individual variation in spontaneous activity and caloric expenditure.
- Most dogs had very strong drive to chew anything available, and this led to early concerns about ingestion of toys and even the resting surfaces. In order to provide some environmental enrichment while in the pens, we identified two toys that resisted destruction by chewing.
- Many of the dogs developed sore foot pads and/or inflammation of the interdigital skin. This was probably due in part to difficulty in keeping runs dry, and consequently failure of pads to dry out and toughen, but also was related to dogs' activity level when given opportunity.
- There were periodic reports of regurgitation or vomiting. In most cases, episodes could be associated with recent exercise, eating or drinking, and were not associated with other clinical signs.
- Shortly after arrival, two dogs (Honey, Rip) were reported for distended abdomens. (Honey had a similar report in the medical record from K2.) Although these were transient events, concern over possible gastric distension (bloat) in the colony prompted use of large stainless steel balls ("Portion Pacer" balls) in all food bowls to slow eating.

Gastrointestinal problems:

- Approximately 50% of the dogs transferred to NCSU developed loose stool/diarrhea within one week of arrival. This was initially attributed to the stress of transport, and change in environment, but reports continued. Over the next month, there were repeated reports of loose or watery stool, occasionally with blood and/or mucus; dogs were consistently without other clinical signs. Initial fecal examinations were negative, and several dogs were treated with a course of metronidazole without significant improvement. Subsequently, a pooled stool sample was evaluated (Idexx Laboratories, Canine Diarrhea Panel) and found to be positive for giardia and coronavirus. (Dogs housed at K2 have been diagnosed with giardia previously.) All dogs were treated with fenbendazole (Panacur; 50 mg/kg PO SID for 5 days) and praziquantel/pyrantel pamoate/febantel (Drontal Plus; as per label directions) between January 7 and January14, 2012, including bathing and facility decontamination.
- Additional veterinary work-up on several dogs (Baxter, Wizard, Jimmy) with continuing diarrhea (± blood) was conducted in consultation with Dr. Jody Gookin, a veterinary small animal internist (gastroenterology). Under sedation, several diagnostics were performed, including rectal scrape and colon flush. Dr. Gookin identified a parasite resembling *Pentatrichomonas hominis* on wet mount. Based on these findings, and continued episodes of diarrhea in other dogs, the entire cohort was treated in March-April with metronidazole (25 mg/kg BID for 2 weeks).
- Fecal scoring was continued and indicated little improvement in April. Additional testing

by Dr. Gookin identified an intestinal yeast (*Cyniclomyces guttulatus*) in several of the dogs. One (Bullet) was treated in May with nystatin (50,000 IU/kg PO for 4 days) as a trial. There was no significant improvement in fecal score two weeks later. Bullet and several other dogs were re-tested and found negative for both yeast and *Pentatrichomonas.*

- Fecal scoring in June showed an overall improvement in the colony, with only a few dogs having unformed or watery feces on an occasional basis.

Other specific health problems:

- Wizard had the most severe diarrhea, with bloody stool reported frequently until recently; he never had a fever or other clinical signs. In addition to the treatments above, he received a course of amoxicillin (400 mg PO BID for 14 days) in February for possible clostridial enteritis. In May, his diet was changed gradually to Purina ProPlan Performance, and his fecal scores were mostly normal in June.
- Baxter had an elevated rectal temperature (> 104°F) shortly after arrival at the NCSU facility He had diarrhea, but findings from physical examination were limited to facial and foot lesions that appeared to be of chronic origin. These lesions were biopsied and the pathologist's report confirmed the presence of skin ulceration and inflammation. Results of an in-house "tick panel" and Leptospira titer were negative. The fever persisted intermittently for several weeks, but the cause was not determined. The case was resolved after treatments with metronidazole and carprofen, and subsequently enroflaxacin and carprofen.
- Dakota developed skin lesions in February; there was patchy alopecia, more prominent on ventrum and limbs, with areas of skin inflammation, crusting and scabbing. Examination for ectoparasites was negative, and initial treatment included medicated baths (Dermachlor-K) and cefpodoxime antiobiotic (Proxetil; 200 mg PO BID for 14 days). Improvement was minimal and, although mites were not seen, she was treated with topical Revolution® (selamectin) every 2 weeks for 3 treatments. After continued minimal improvement, we obtained a consult from Dr. Thierry Olivry, a veterinary dermatologist, who recommended further treatment for bacterial dermatitis (cefovecin (Convenia) long-acting injectable, 8 mg/kg for two doses, plus medicated baths) but also suggested possibility of atopic dermatitis. After minimal improvement, Dakota was treated with two courses of prednisone (15 mg PO SID for 14 days), which resulted in marked improvement. Based on a presumptive diagnosis of atopic dermatitis, we started a novel protein diet trial in April (Iams kangaroo/potato), and most clinical signs were resolved by late June.
- Mercy was reported in April for patchy alopecia on ventrum and limbs, with some crusting and scabbing. Ectoparasites were ruled out, and diet change (to Purina ProPlan Performance) was tried. Skin condition improved gradually and was normal by the end of May.
- Reno suffered a short apparent seizure on May 29, 2012. He was found by an animal care technician on his side (lateral recumbency), with legs rigid and chomping; the technician thought he was somewhat responsive to voice, and that this went on for no more than 5 minutes. On arrival of the LAR veterinarian, Reno looked normal, and physical examination was unremarkable except for an elevated rectal temperature (103.8°F); blood glucose by stick was 97 (normal). Blood was collected and submitted to rule out other metabolic causes of seizure; the blood work was unremarkable. Our working diagnosis is primary epilepsy. Although an infectious or structural (neoplasia, vascular event) cause is possible, our veterinarians thought these unlikely given the quick resolution of signs, lack of other clinical signs, and his age. Additional treatments were

not recommended, and there has been no recurrence of clinical signs.

Chemicals (Odorants)

Several test odors were used, including vanillin, ammonium nitrate (AN), and AN combinations. Several AN formulations (purified- and fertilizer-grade) were used. Ammonium nitrate and other test odors were presented to dogs in suitable containers for field studies (e.g., nylon mesh or PVC containers) or, in laboratory studies, were contained within PVC or other plastic closed containers. Direct contact of dogs with the test odors did not occur at either NCSU or K2. The ammonium nitrate-based fertilizer was purchased from Weaver Fertilizer (Winston-Salem, NC). Unless otherwise noted all other chemicals were purchased from Sigma Aldrich Chemical Co. (Milwaukee, WI).

Chemical Name	CAS Registry Number
Ammonium nitrate	6484-52-2
Ammonium nitrate(34-0-0) fertilizer	6484-52-2
Ammonium chloride	12125-02-9
Ammonium sulfate	7783-20-2
Silver nitrate	7761-88-8
Amyl acetate	628-63-7
Sodium sulfate	7727-73-3
Vanillin	8014-42-4

Noise (auditory stimuli)

The noise levels used in this experiment (up to 120 dB) were similar to those encountered with extremely loud music amplifiers (~120 dB), jet engine noise (138 dB at 100 feet), and gunshot/firecracker (140 dB at 2-3 feet). We also considered that the breed used for this study (Labrador Retriever) is a "gun dog" that has been adapted to minimizing responses to gunfire over decades of breeding. As for pain, the human literature indicates that this response is momentary following an acute loud noise exposure. All personnel working with the dogs wore ear protection during these procedures. Additional details regarding the use of auditory stimuli are presented elsewhere in this report.

Statistical Analysis

All data were visually inspected before analysis. Some data were identified as potential outliers during this inspection. Two methods were used to handle potential outliers: (a) expert-based exclusion based upon experimental conditions (e.g., loss of motivation in a dog during a test session); and (b) statistical tests (Dixon's test). Some data were censored (e.g., time to detect odor, salivary cortisol concentration). For example, in some of our field studies we gave the dogs a maximum time of 180 seconds to find the source of odor in an approximately 40 m X 40 m field. For data analysis purposes, a value of 180 (i.e., right censored) was assigned when the dog was "timed out". In the case of plasma cortisol concentration, a value of 0.99 μg/dL was used when the sample concentration was below the limit of detection (< 1.00 μg/dL). Because the group size for spayed versus intact female dogs was small, all female dogs were analyzed collectively irrespective of their reproductive status.

Levene's test for homogeneity followed by one-way analysis of variance (ANOVA) ($p < 0.05$) and Dunnett's t-test were performed for continuous data. In the event that the Levene's test on the transformed data indicated non-homogenous data, a Kruskal-Wallis H test and Wilcoxon 2-

sample Rank-Sum test were sometimes used. Depending upon the data set, an adaptation of the Student's t-test (i.e., Welch's t- test) intended for use with two samples having possibly unequal variances was used. Categorical data were converted to ordinal scores and analyzed using a contingency analysis. Significant changes were further analyzed using a log-likelihood model and Pearson's Chi Square. Some data were also analyzed using appropriate statistical models testing the impact of multiple factors (e.g., sex, trial number, etc). When a factor was identified as not statistically significant, the data were pooled appropriately (e.g., no effect of sex). Pearson's correlation tests were used to assess the strength of a linear association. For all statistical tests, JMP 9.0 (Cary, NC) was used, and the results were considered significant if $p \leq 0.05$. Unless otherwise indicated, data presented in all figures represent mean values ± standard error of the mean (SEM).

SECTION II: EXPERIMENTAL STUDIES CONDUCTED AT NCSU

PHASE I. EMOTIONAL REACTIVITY TEST (ERT)

Background

Military working dogs specialized as Improvised Explosive Device Detector Dogs (IDDs) have been described as "over and above the best tool available" to detect explosives in operational environments. However, training prospective IDDs is costly in terms of time, effort, and resources. Because the performance of deployed IDDs may have life or death implications, only the most physically and emotionally capable dogs should be selected for training as IDDs. Therefore, it is imperative to develop methods to determine the temperamental and behavioral suitability of dogs for IDD work at the earliest stage, prior to the initiation of IDD training.

One component of IDD-specific behavioral competence is the ability to respond minimally and recover quickly from environmental unpredictability. As living organisms, IDDs are susceptible to a range of behavioral "stress" effects that may negatively impact their functional capacity and welfare. Stress effects may be amplified by extreme working conditions, catastrophic events in the field, and variability in handler competence. Reduced performance may have significant negative impact on the health and well-being of military personnel.

In addition, fear and anxiety in dogs can negatively impact learning capability and performance of learned tasks. Such emotional responses may arise from heritable temperament, genetic traits, early experiences, conditioned responses, or combinations of these. Selecting dogs for military olfactory detection work that are robust to fear responses will optimize learning, improve performance, and reduce "stress" responses in the field. The behavioral response to novel environment stressors may differ among dogs depending on the types of stimuli presented and differences in temperament among dogs (Rooney et al., 2007).

Standardized tests are used to evaluate young working and guide dogs prior to task-specific training (Duffy and Serpell 2012, Sinn et al 2010). Guide dogs, like IDDs, represent a specialized form of working dog. The primary reason for disqualification of guide dogs is commonly reported to be lack of behavioral suitability (Arata et al., 2010; Serpell and Hsu, 2001). Researchers have attempted to predict the future capabilities of candidate guide dogs with temperament assessments including questionnaire surveys and behavior tests. Behavioral testing typically involves exposing dogs to a limited range of controlled test situations that are considered useful for evaluating the behavioral traits of interest (e.g. aggression, fear, confidence, trainability, and so on), and assigning scores to their responses. One advantage of this testing is

that all dogs are exposed to identical test situations and are scored by the same trained observer(s).

Early testing of candidate IDDs is also important. There are four primary areas in which the dogs are evaluated prior to selection and entry into the IDD training program. They are evaluated for hunt behavior, hunt training (directional control), emotional reactivity, and good health, as determined by medical screening. Dogs must pass all portions of the test battery prior to entry into IDD training.

To evaluate emotional reactivity, the USMC IDD program has developed the Emotional Reactivity Test (ERT) for the evaluation of each dog's: (a) sensitivity to unusual or loud sounds; (b) sensitivity to sudden and unexpected sights; (c) threshold of emotional reactivity; (d) speed and degree of recovery from emotional reaction; (e) willingness to interact with strangers; and (f) behavioral constancy and task perseverance. These features are incorporated into a series of standardized challenges, called tasks. In response to each task, a dog is assigned a score, ranging from 1-5 in response to each task. In general, a lower score indicates greater emotional reactivity (fear) than a higher score. In spite of its purpose, the predictive validity of the ERT to select dogs robust to "stress" effects has not been previously evaluated. One goal of the present study was to evaluate and refine the U.S. Marine Corps Emotional Reactivity Test for detection dogs to optimize dog selection in order to identify and reject dogs that are emotionally labile, and to select the most resilient dogs for training and deployment.

In Phase I, we examined the association between temperament traits (as assessed using an NCSU-adapted version of the ERT) and a dog's stress/anxiety response to novel stimuli. This was accomplished in two ways: (a) measurement of salivary and plasma cortisol concentrations prior to and after the ERT, and (b) anxiety responses to neophobic stimuli presented in an open field model (Phase II). Changes in cortisol levels are well documented as a major physiological response to stress (Coppola et al., 2006), and both salivary and plasma cortisol samples were collected in Phase I. Both salivary and plasma cortisol samples were collected in Phase I. Cortisol in saliva is unbound and is present at approximately 7-12% of plasma concentrations (Beerda, et al., 1996; Beerda, et al., 1998). The use of saliva for evaluation of cortisol allows for a fast, non-invasive method of sample collection. However, plasma cortisol may offer a more rapid, consistent and sensitive measure.

The overall goals of this phase were to evaluate the reliability and predictive nature of the USMC ERT and a modified version of the ERT developed by NCSU (NCSU ERT), and to evaluate the ERT for its ability to select dogs that are most suitable for training and deployment. In this manner, we hoped to reveal traits that would serve as targets for predicting future IDD performance. Data analysis presented elsewhere in this report will examine whether the ERT results were associated with performance on tests of canine olfaction and cognition (Phases III to V).

Materials and Methods

ERT Facilities and testing dates

Soon after being received at K2, months prior to Phase I experiments at NCSU, K2 staff conducted the K2 version of the USMC ERT on some, but not all of the 28 dogs in the present study (Cohort I and Cohort II). Completion of the K2 version of the USMC ERT protocol was performed over one or more days of testing for each dog. Results of the K2 USMC ERT were made available to NCSU scientists for comparison with NCSU tests.

NCSU scientists performed the NCSU Adapted ERT at NCSU (Cohort 1) on February 28, 2012 and at K2 (Cohort 2) on June 5, 2012. To reduce handler bias, a single handler (Dr. Margaret Gruen) was used for all dogs. 'Real-time' assessments were performed by a single assessor (Ms. Beth Case) for Cohort 1; 'real-time' assessments were performed by two assessors (Ms. Beth Case and Dr. Barbara Sherman) for Cohort 2 in order to evaluate inter-observer variability. In addition to the 'real-time' scoring of observations, all ERT components were captured by video recordings for further review and verification of scores. One video camera captured all outdoor assessments, and two video cameras captured all indoor assessments.

Portions of the ERT were performed outdoors under ambient environmental conditions. Other portions were conducted indoors. The facilities at the two sites (NCSU and K2) differed appreciably, and the tasks, such as reaction to stairs, were modified as necessary. For example, at NCSU, a small flight (5 steps) of wooden stairs (Figure 2) was utilized. The top of the stairs opened onto a small (1.14 m^2) platform with an open metal floor followed by a steep (24°) 2.34 m long downward ramp. In contrast, this task performed at K2 involved a full flight (1 floor) of an open-grate metal stairway that opened onto a platform with subsequent entry into the indoor test facility. Likewise, certain tasks, such as the dog's response to a gunshot outdoors, were modified based upon the testing facility. At K2 this criteria was met using a gun modified to use a 0.32 caliber blank. At NCSU, in lieu of a firearm discharge, we assessed the dog's reaction to audio recordings of a shotgun discharge at two peak sound levels (mean of 102.2 and 110.6 dBA SPL), performed indoors. This modification was required because of the proximity of the NCSU canine facility to other laboratory animals housed in the LAR facility which might be unduly stressed by an outdoor firearm discharge. However, for most tasks, the tests were similar at both sites.

Our goal was not to standardize the test apparatuses used, but rather to evaluate how robust the ERT was under very different testing conditions.

Figure 2. Screen capture images from video recordings showing the outdoor stairs and ramp built for the NCSU version of the ERT. Dr. Gruen is shown working the dogs. Bottom figure shows close up reactions of a second dog to the flight of stairs.

Overview of ERT protocols

Several versions of the ERT are referred to in this report and are summarized below. This is due to the different versions used by K2 and NCSU, and test enhancements added to improve the sensitivity of the test to stratify dogs with regard to anxiety. The NCSU ERT was adapted from the USMC ERT to increase the sensitivity for detection of anxiety. Several novel components were added (e.g., dog's reaction to an opening umbrella or a radio controlled car [see Figure 3 below]). Although these elements are not included in the original USMC ERT, they are included in temperament tests used by other organizations (e.g., Guiding Eyes for the Blind). Scores for each component (task/subtask) of the ERT were assigned based on a 5 point scale (Table 3) and were recorded at the completion of each task.

Test name	Number of subtasks	Total points	Comments
USMC ERT	12	60	Original test, Used by K2
NCSU USMC ERT	12	60	Subset of scores from NCSU ERT that match the USMC ERT test performed by K2
NCSU ERT	25	125	Performed by NCSU at NCSU
NCSU ERT (K2)	23	115	Performed by NCSU at K2 (reflects the changes made due to facility differences)
NCSU ERT Anxiety Score	20	100	Excludes scores for subtasks not directly related to anxiety

Figure 3. Screen capture images from video recordings showing the indoor facility used for the NCSU version of the ERT. Dr. Gruen is shown working dogs as they respond to a radio-controlled vehicle. Grates can also be seen to Dr. Gruen's left. Audio recordings of a shotgun discharge were presented in this room as part of the NCSU ERT.

Overview of NCSU ERT protocol

Tasks in the NCSU version of the ERT were administered as a continuous sequence. In all cases, if the dog showed excessive fear in response to a task, then that task was discontinued. The test area and people involved were unfamiliar to the dog. The dog handler (Dr. Gruen) had extensive dog experience. The handler quietly and gently guided the dog through the tasks of the test without undue restraint. For each task, the handler allowed the dog to approach the task object or person independently. If the dog did not approach independently the handler then encouraged the dog to approach. The NCSU version of the ERT includes all elements included in the USMC ERT but as previously noted includes additional elements. The NCSU ERT is composed of the following tasks, many of which included subtasks with regard to scoring:

1. Stairs / Surface Up & Down- Location: outdoors
   The dog is walked up a flight of open back stairs (at NCSU, dog was also walked across a metal grate surface). Following other subtasks, the dog is then walked down the same flight of stairs. The dog's willingness to walk up and down the stairs is scored
2. Crowd - Location: outdoors.
   The dog is walked through a crowd of 4 to 5 people two times (once through the crowd, then turned, and walked back through the crowd again). The dog's reaction to the crowd is scored.

3. Stranger Exam - Location: outdoors (Performed twice during test)
   Handler and dog approach a stranger who performs a cursory examination on the dog (hands run along dog's body). The dog's reaction to the exam and willingness to approach the stranger are scored.
4. Visual Startle (Bag Drop) - Location: indoors.

A bag containing newspaper is dropped in front of the dog as it is walking. The dog's reaction is scored, as well as its willingness to approach and investigate the bag.

5. Acoustic Startle (Metal Grates) - Location: indoors
   A metal grate is dropped in front of the dog as it is walking. The dog's reaction is scored, as well as its willingness to approach and investigate the grate. As the dog is walked away, a second metal grate is dropped and the dog's reaction is scored.
6. Unusual Stranger - Location: indoors
   An unusual stranger (person wearing a sheet or burqa) walks slowly toward the dog from 50 feet away, approaching to within 30 feet of the dog (stopping at 10 foot increments. The dog is then allowed to approach and greet the unusual stranger. The dog's reaction to the stranger, any aggression observed, and the dog's willingness to approach and greet the stranger are scored.
7. Umbrella Startle - Location: outdoors
   A person holding an automatic umbrella opens the umbrella quickly when the dog is within 3 feet. The dog's reaction to the umbrella opening is scored.
8. Remote Control Vehicle - Location: indoors
   A remote control vehicle is driven out from behind a barrier toward the dog, and is moved back and forth 2 times. The dog's reaction is scored, as well as its willingness to approach and investigate the vehicle.
9. Gunfire (recorded at NCSU) - Location: indoors at NCSU, outside at K2
   The dog is exposed to gunfire and its reaction is scored.

NCSU ERT data analysis

Categorical data were converted to ordinal scores as noted in Table 3 and analyzed using a contingency analysis. Significant differences were further analyzed using a log-likelihood model ($p < 0.05$) and Pearson's Chi Square.

Salivary and plasma cortisol

Baseline saliva and blood samples for cortisol measurement were collected from cohort 1 in the afternoon (approximately 1400-1600 pm) on February 22, 2012 (a week prior to ERT testing). Saliva and blood were also collected within 10-15 minutes after the end of the ERT. All of these sample collections were in the afternoon, at least 2 hours after a meal.

Dogs were first trained for approximately one week to allow one end of a cotton rope to be placed in their mouth while the experimenter held a small piece of treat (Pup-peroni®, DelMonte Foods) in a closed hand in front of the dog. The dog was encouraged to sniff the treat to stimulate salivation. After collecting an adequate sample volume within 2-3 minutes, the dog was given the treat. This method reduced the amount of restraint needed and facilitated collection of an adequate volume (> 0.3 mL) in a small amount of time (Bennett and Hayssen, 2010).

Saliva was collected with a 7-cm piece of cotton rope (Salimetrics, State College, PA) at least 2 hours after a meal. The wet end of the rope was placed in a centrifuge tube and kept on ice until the sample could be extracted. Within 4 hours of sample collection, samples were centrifuged at 4 °C for 20 minutes at 1300 g to extract the saliva from the rope. Saliva (0.1 – 1.8 ml) was transferred to a microfuge tube and stored at -20 °C until analysis. Blood was collected immediately after the baseline and post-ERT saliva collections. Blood (4-6 ml) was collected from the cephalic vein, using a butterfly catheter and vacutainers with EDTA. Blood was centrifuged at 4 °C for 15 minutes at 1300 g to separate the plasma. The plasma was removed by pipette and placed in microfuge tubes for storage at -20 °C until analysis.

Cortisol concentrations in saliva were assayed in duplicate using a Salimetrics high sensitivity salivary cortisol enzyme immunoassay (EIA) kit (State College, PA). It is a competitive immunoassay with a limit of detection of 0.03 µg/dL. Plasma cortisol was measured using an Immulite 1000 Cortisol kit (Siemens Healthcare Diagnostics, Tarrytown, NY), with a limit of detection of 1 µg /dL. The laboratory range for canine plasma resting cortisol is 1.0-4.5 µg /dL. The Immulite kit is a solid-phase, competitive chemiluminescent enzyme immunoassay.

Results

NCSU ERT responses were scored in real time, and verified or altered by review of video recordings. When scored by 2 observers (Dr. Sherman and Ms. Beth Case), scores for 76.1% of subtasks were identical, and the average difference in total scores between observers was 2.5 points. Results of the NCSU ERT testing in cohort 1 and 2 are presented in Tables 4 and 5, respectively. Mean (± SEM) total NCSU ERT scores were 105.9 ± 2.2 and 94.6 ± 6.1 for male and female dogs, respectively. This difference was not statistically significant ($p = 0.3453$, Pearson's χ^2). Mean (± SEM) total NCSU ERT scores were 104.6 ± 2.2 and 93.0 ± 7.9 for black- and yellow-coated dogs, respectively. This difference was not statistically significant ($p = 0.5270$, Pearson's χ^2).

We found that the USMC ERT scores generated by the K2 staff and the NCSU research team were strongly correlated (Figures 4 and 5). With one exception ("Piper") in cohort 1, the USMC ERT values are relatively stable ($p < 0.05$; $r^2 = 0.672$, data analysis excludes one dog [Piper]) despite having been performed by different individuals, locations, and times. As a test-retest measure, we found a similar result with the USMC ERTs performed on cohort 2. Several dogs in cohort 2 were unable to complete the ERT due to profound fear responses to certain subtasks. As with cohort 1, we saw a significant association between these two scores (Figure 5; $p < 0.05$; $r^2 > 0.9$).

Figure 4. Comparison of results from the NCSU and K2 versions of the USMC ERT (Cohort 1).

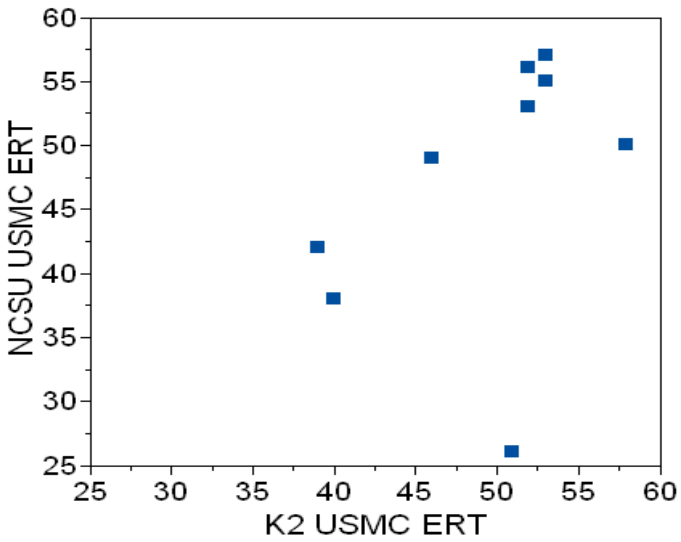
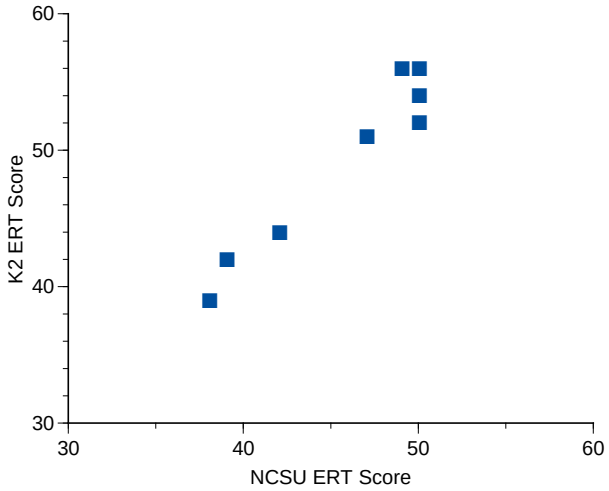
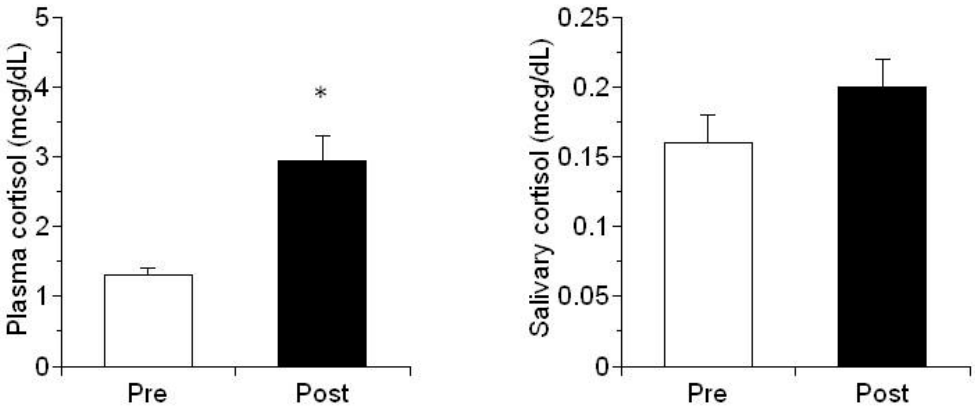


Figure 5. Comparison of results from the NCSU and K2 versions of the USMC ERT (Cohort 2).



As mentioned earlier, pre-ERT blood and saliva cortisol concentrations represent basal levels collected approximately one week prior to the conduct of the ERT (Cohort 1 only). Post-ERT values were determined in blood and saliva samples collected within 10 to 15 minutes of the completion of the ERT (Table 8). A statistically significant increase in plasma cortisol concentration was seen following completion of the NCSU ERT (Figure 6). Salivary cortisol concentration seen in dogs following the ERT was not significantly increased (Figure 6; $p = 0.0686$).

Figure 6. Plasma (Left) and saliva (Right) cortisol concentration in dogs measured before and after completion of the NCSU ERT (* $p < 0.05$).



The relative change in salivary cortisol concentration was also evaluated (% change versus baseline [pre-ERT] values). There was no significant effect of sex or coat color on the % change in plasma or salivary cortisol. Changes in both salivary and plasma cortisol concentration were correlated with total NCSU ERT score (Figure 7).

Figure 7. Correlation seen between ERT and relative increase in plasma (Left) and saliva (Right) cortisol concentrations in dogs.

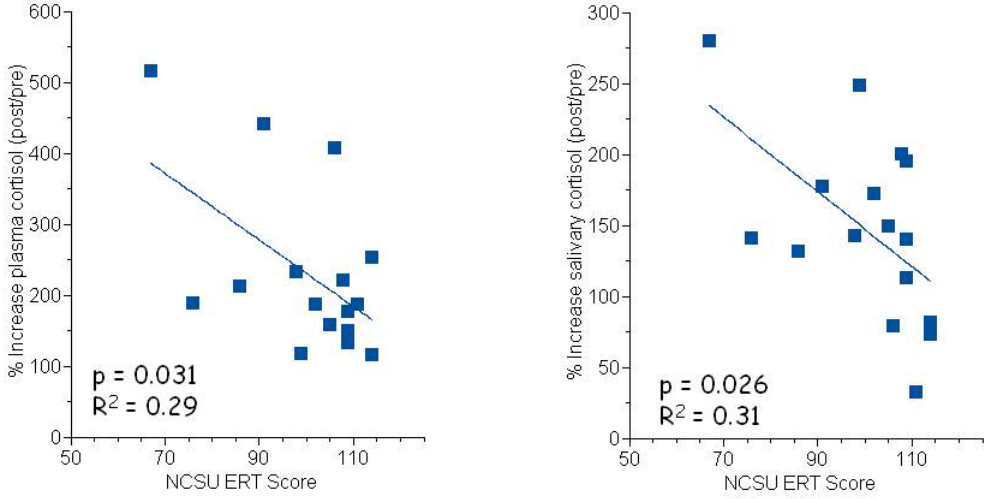
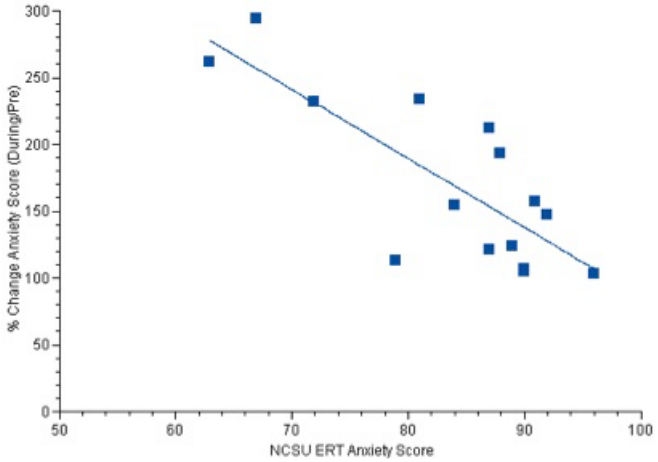


Figure 8 shows the linear correlation between NCSU ERT anxiety score and the % change in the open field anxiety score (100% means no change). If we include Piper the p value was 0.0299 with an r^2 value of only 0.294. If we exclude Piper then the p value was 0.0005 with an r^2 value of 0.62 (Figure 7). See Phase II (Development of an Open Field Anxiety Test) for additional information about the open field anxiety score.

Figure 8. Positive correlation between ERT and normalized anxiety scores. Data analysis excludes one dog ('Piper').



Based on the results of the open field anxiety scores (Phase II), dogs were categorized into two groups: those that had the greatest change in anxiety score during treatment periods ("worst" dogs, n=8), and those with a smaller change in anxiety score during treatment periods ("non-

worst" dogs, n=8). Further examination of these two groups (Table 15 – see Phase II) revealed that the "worst" dogs had:

- lower overall ERT scores with a mean of 75.4 versus other dogs' mean of 88.4 (t-test with unequal variances; p=0.0308, t=2.56)
- lower ERT scores on collection of 4 tests of Acoustic Startle (sum score of 3 grate tasks and first gunfire task) with a mean of 12.87 versus other dogs' mean of 17.87 (t-test with unequal variances; p=0.0032, t=3.91)
    - lower ERT scores on the sum of the grate (acoustic startle) tasks with a mean of 9.25 versus other dogs' mean of 13.5 (t-test with unequal variances; p=0.0051, t=3.66)
    - lower ERT scores on the first gunfire task with a mean of 3.62 versus other dogs' mean of 3.38 (t-test with pooled variances; p=0.0346, t=2.34)
- lower ERT scores on test of Visual Startle (bag drop) with a mean of 7.00 versus other dogs' mean of 9.62 (t-test with unequal variances; p=0.050, t=2.32)
- larger change in plasma cortisol post-ERT with a mean of 2.35 versus other dogs' 0.90 (t-test with unequal variances; p=0.0270, t=2.47)

Discussion

We adapted the USMC ERT to increase its potential for dog evaluation, and successfully performed the NCSU USMC ERT on two cohorts of dogs, those housed at NCSU (Cohort 1, n=16) and those housed at K2 (Cohort 2, n=12). We utilized the NCSU USMC ERT results, in combination with the results of the open field anxiety tests (Phase II), to improve and validate the ERT as a useful and important IDD screening test. It is critical that the ERT be conducted in a systematic manner using trained observers. We found that videotaping the ERT allowed for post hoc analysis of the test.

In order to determine the predictive value of the ERT, we established four quality requirements: standardization, consistency, sensitivity, and validity (Sinn et al 2010). Standardization referred to consistency of the test stimuli, notation, and scoring and ensured that each dog received a specific test, scored in a consistent manner. As elucidated above, the ERT protocol was standardized with improved scoring definitions such that different trained individuals scored the tests similarly. The test-retest measures were consistent. Good inter-observer reliability was obtained for standardization.

Consistency referred to the fact that when given to individual dogs more than one time, scores were repeatable. Consistency was measured by test-retest measures with a significant correlation between two exposures of the USMC test to dogs in Cohort 1 and Cohort 2. These results also show that the USMC ERT is stable across time and generally reflects behavioral characteristics of each dog that are not significantly modified by experience/training.

Sensitivity referred to the ability of the ERT to elucidate behavioral differences between dogs and stratify a sample population of dogs based on behavioral differences. As shown in the results, dogs were behaviorally stratified based on their ERT score. The test was sensitive and elucidated behavioral differences between the dogs. We expanded the sensitivity of the test and its ability to detect differences between dogs (125 points) and added novel challenges and repeat measures.

Validity referred to the ability of the test to accurately measure specific behavioral traits and predict dogs' future behavior with regard to emotional responses. Cortisol levels were used to demonstrate the stressful nature of the emotional reactivity test by comparing baseline plasma and

baseline salivary cortisol levels to post-ERT values. There was a significant increase in post-ERT plasma cortisol concentration compared to baseline. The plasma cortisol concentration seen after the ERT is within our laboratory's normal reference range. As in other studies, cortisol levels may be useful measures of the response of working dogs to environmental challenges (Haverbeke et al 2008). Although some other studies have shown an increase in salivary cortisol in response to different types of stimuli in dogs (Beerda et al. 1998), salivary cortisol is not as sensitive a measure as plasma cortisol.

Predictive validity of the ERT test was confirmed by the correlation with the open field percent change anxiety score (see Phase II for details). Based on the mean anxiety scores generated in the open field challenge tests, dogs fell into two groups, designated "Worst" and "Non-Worst." The ERT scores of the "worst" dogs were significantly lower than those in the "non-worst" category. Scores of worst dogs were significantly lower than those of "non-worst" dogs on 5 subtests. These subtest scores in combination with the total ERT scores may be used to identify dogs at risk for fear responses. On this basis, ERT task scores and total scores could be used a priori to identify dogs susceptible to fear responses.

Thus, the results of this study demonstrate the usefulness of the canine Emotional Reactivity Test in evaluating the suitability of individuals for subsequent IDD training and deployment. In general, dogs with low (reactive) scores on the ERT showed high anxiety scores in the open field model. Based on open field anxiety scores, dogs were classified as "worst" or "non-worst." The ERT scores of "worst" dogs were significantly lower and the plasma cortisol levels significantly higher than those of "non-worst" dogs. In addition, "worst" dogs showed lower ERT scores on ERT startle tests, quantifying the predictive validity of the test and its usefulness in evaluating candidate IDDs.

In conclusion, the U.S. Marine Corps Emotional Reactivity Test (ERT) was modified by NCSU scientists to screen dogs for emotional reactivity and resilience, resistance to stress effects, and rapid and complete recovery from environmental challenges (tasks). Based upon our experience conducting the test and evaluating dogs' responses, modifications were made including adding tasks, subtasks, and score descriptions to consistently capture performance and interpretation of the ERT. The test battery evaluates a dog's response to a variety of stimuli, including novel substrates, unfamiliar persons, loud sounds and novel visual objects. NCSU Phase I research has established predictive validity of the NSCU USMC ERT and its usefulness in the process of eliminating from training dogs susceptible to fear responses for IDD training.

PHASE II. DEVELOPMENT OF AN OPEN FIELD ANXIETY TEST

Background

As mentioned earlier in Phase I, fear and anxiety in dogs can negatively impact learning capability and performance of learned tasks. The anecdotal reports of so-called canine post traumatic stress syndrome (Walter F. Burghardt Jr., personal communication, 2012) in IDDs and other military working dogs provides evidence for the intensity of the work environment in which these animals serve, and suggests that dogs vary in susceptibility to fear responses. Identification of dogs with the emotional resilience to cope with environmental stress is therefore an important selection criterion for candidate IDDs.

The correlation of dogs' emotional responses (ERT, Phase I) in repeated measures over time and in spite of training, suggests that fear and anxiety may be intrinsic to individual dogs. Under certain circumstances, a fear response may be adaptive; however, in some animals an exaggerated maladaptive stress response may occur, particularly in stressful environments. In simplest terms, anxiety is the anticipation of danger, usually from unknown or imagined origin. Fear is the anticipation or awareness of danger, which is termed a phobia if specific to a certain modality, such as noise. Anxious animals may be hypervigilant even in the absence of specific stimuli and may startle easily, assume low posture, or show more subtle, but observable, signs of yawning, tongue flicking, or lip licking. They may also exhibit specific physiologic responses. Fear and anxiety can result in enhanced activation of the hypothalamic–pituitary–adrenal axis with subsequent release of cortisol, noradrenalin, and adrenaline. Prolonged stress-induced activation of the hypothalamic–pituitary–adrenal axis is a known risk factor for certain gastrointestinal, dermatologic, immunologic, and urinary tract disorders in dogs (Beerda et al., 1999; Gue et al., 1987, Hydbring-Sandberg et al., 2004) and may represent a welfare concern.

In Phase I, we showed that the novel human and environmental challenges that form the basis of the Emotional Reactivity Test (ERT) induce stress responses in candidate IDDs. ERT-induced stress resulted in behavioral (e.g., fear and anxiety) and physiological (i.e., increased salivary and plasma cortisol concentrations) responses. A well-conducted and applied ERT can therefore exclude many candidate IDDs. Phase II further validates the use of the ERT as a selection tool for candidate IDDs. In this research phase we examined the responses of dogs to a novel sound stimulus in an open field model.

In dogs, noise phobia involves the expression of excessive fear in response to a sound stimulus (Sherman and Mills 2008, Crowell-Davis et al., 2003). Investigators have used audio recordings of thunderstorms in controlled environments to assess sound phobias in dogs (Araujo et al., 2009; Araujo et al., 2010; 2010; Shull-Selcer and Stagg, 1991). These playback experiments are designed to provide an objective measure of the dogs' reactions to acoustic stimuli. Although differences exist between a playback of a sound recording and the actual events of thunderstorms, fireworks, and other sound stimuli, this technique has been used to categorize the reactivity of dogs to sounds and to desensitize and counter condition dogs with noise phobia (Overall, 2002).

The use of an Open Field Test (OFT) has a long history in experimental psychology (Walsh and Cummins, 1976). The OFT was originally developed to assess general locomotor activity levels and anxiety in rodents. The open field used in rodent studies is often a small enclosure that allows for animal observation while containing the animal safely in a confined space. Infrared photobeams, computer-based tracking systems, and other approaches are used to assess animal movement. When anxious, the natural tendency of rodents is to prefer staying close to the walls

(thigmotaxis). In this context, anxiety-related behavior is measured by the degree to which the rodent avoids the center of the open field. Adaptive exploratory behaviors are also seen in the open field. The basic principles and design of the rodent OFT have been adapted for use in other species including dogs (Araujo et al., 2009; Siwak et al, 2003; Head et al, 1997).

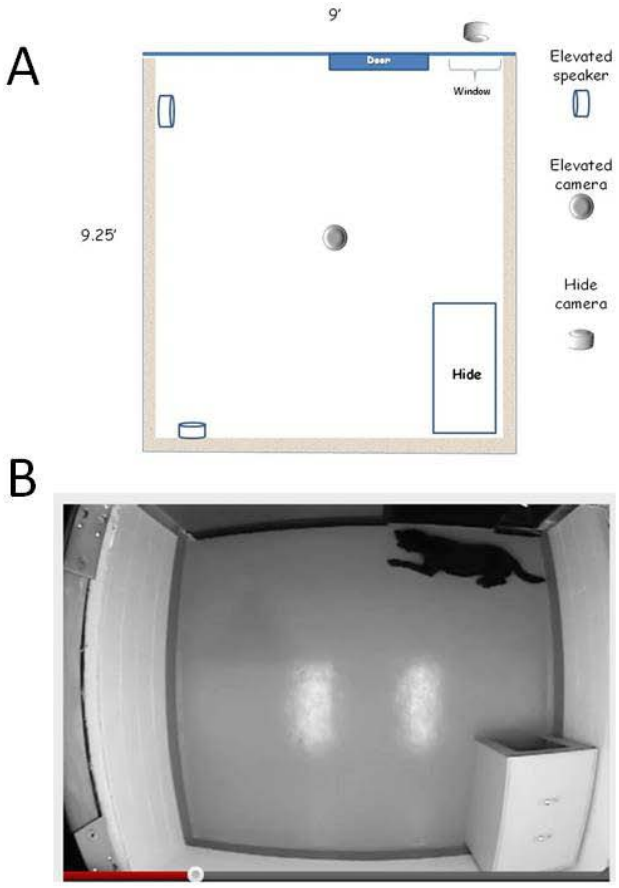
The overall goals of Phase II (Cohort 1 only) were to create a "stress" model in order to further characterize the behavioral responses of Labrador Retrievers when they are subjected to a high-intensity neophobic sound stimuli, and to validate the ERT test. The neophobic stimuli we used in this experiment involved the presentation of audio recordings of thunderstorms and simulated gun battle.

Materials and Methods

Open-field arena

The open field arena consisted of a room approximately 2.9 X 2.7 m (Figure 9). The open field had three cinder block walls with a fourth modular wall. The open-field arena was equipped with an open-floored 61 x 76 x 91 cm (W x H x L) hide constructed of high-density polyethylene sheets (King StarBoard®, Piedmont Plastics, Morrisville, NC). The open-field arena had a camera mounted in the center close to the ceiling level. A second horizontally mounted camera with an infrared filter and illuminator recorded the dog's behavior while in the hide. The open-field arena was sanitized with Virkon®-S (Dupont, Fayetteville NC) diluted to 0.25% strength and applied to the floor to reduce olfactory cues from the previous test subject. Each dog was placed into the arena for 9 minutes per test and the test session was recorded digitally using EthoVision XT software (Noldus Information Technology, Leesburg, VA).

Figure 9A. A schematic representation of the NCSU open field test arena. Approximate location of speakers, cameras, and hide are shown. Not to scale. Figure 9B. Screen capture images from video recordings showing a dog during an open field session. The Noldus EthoVision XT system is used to calculate the dog's movement and posture in the open field arena in response to auditory stimuli. Note: the distorted image of the video is the result of the wide angle lens used in the Noldus system.



Noise stimuli

Audio recordings of the sounds of thunderstorm (CanCog Technologies, Toronto, Ontario) or simulated gun battle (K2, Southern Pines, NC) were played to dogs while in the open field arena. Background sound level (without a dog) was approximately 46-50 dBA, SPL. The mean thunderstorm sound level used was 88.8 dBA, SPL (sound exposure level [SEL] = 110.9, peak = 104 to 105 dBA). The mean gun battle sound level used was 95.2 dBA, SPL (sound exposure level [SEL] = 117.2). The available literature suggested that our sound exposure would induce stress (e.g., behavioral changes and altered cortisol) in some, but not all dogs (Hydbring-Sandberg et al., 2004). However, the sound exposure was not intended to produce distress (i.e., an aversive, negative state in which an animal's coping and adaptation responses fail to return the animal to a state of normal physiological and/or psychological well being (NAS, 2008).

Open Field Test (OFT)

Open field-testing was started on March 5, 2012 and was completed on March 16, 2012. Half the subjects were tested each day, Monday through Friday, of week 1 and half were tested Monday through Friday on week 2. The order of the dogs was initially randomized for each group (week 1 and week 2), then dogs were tested in the same order each day. Each dog was placed in the open field for 9 minutes on 5 consecutive test days. The 9 minute period was divided into three 3 minute epochs. The first and last 3 minute epochs on each day had no auditory stimuli (quiet) while the middle epoch could either be quiet, or include an auditory stimulus (thunderstorm or gun battle sounds). The following open field test schedule was used:

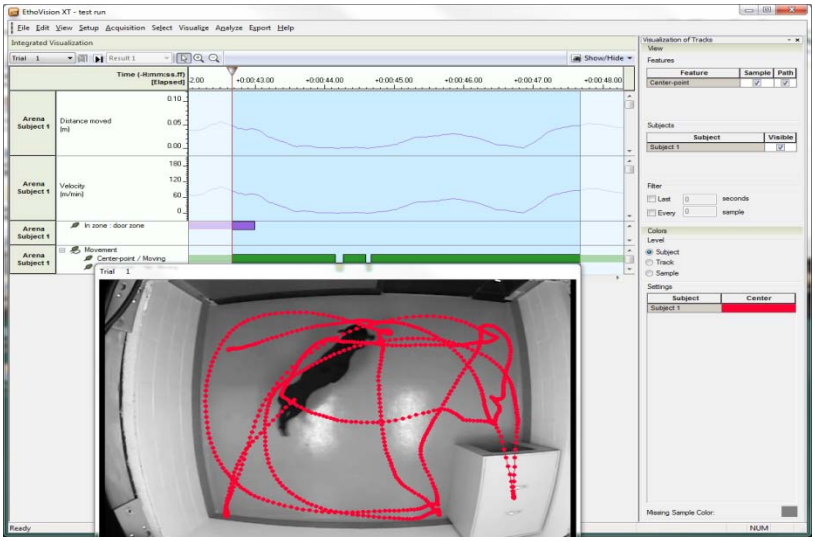
- Day 1: No sound - 9 minutes
- Day 2: 3 min quiet, 3 min thunderstorm, 3 min quiet
- Day 3: No sound -9 minutes
- Day 4: 3 min quiet, 3 min simulated gun battle, 3 min quiet
- Day 5: No sound - 9 minutes

The following data were collected for each dog's daily session:

1. Physiological data
    - *Heart rate and rectal temperature*
        - Heart rate and rectal temperature were determined immediately before and after each 9 minute OFT session. These were determined manually by a trained person, familiar to the dogs, using a stethoscope and digital rectal thermometer.
    - *Salivary cortisol*
        - Saliva was collected after each open field trial using a cotton rope placed in the dog's mouth until saturated (see Phase I for methods; baseline saliva and blood samples were also collected from each dog during the week of February 20, 2012).
2. Behavioral data
    - *Distance*
        - The overhead video images were used to track each dog's locomotion within the open field, and distance travelled was determined using EthoVision XT 7.1 software (Noldus Information Technology, Leesburg, VA).

Figure 10 shows a representative trace of a dog's movement during the open field session.

Figure 10. Animal movement recorded during a portion of a 9 minute open field experiment.



Analysis of motor activity

The EthoVision software provides several options for data analysis that are based upon a combination of the following three variables: (a) sampling rate during the acquisition of the track (i.e., how often does the computer examine whether a movement occurs); and/or (b) using a smoothing function after track acquisition; and/or (c) establishing a minimum distance moved filter. Based on discussions with the manufacturer (Noldus) technical representatives and our own data analysis we have opted to not use the Minimum Distance Moved (MDM) filter since it may lead to 'dropped' data points resulting in a artificially low estimate of the total distance moved by a dog. The final data analysis used a sampling rate of 10 samples per second during trial acquisition and used maximum track smoothing after acquisition. Data analysis provided estimates of the following parameters: (a) total distance moved (m), mean velocity (m/min), door zone duration (min), hidden zone duration (min), front wall zone duration (min), and center-point /not moving duration (min). The center-point /not moving duration parameter is manually calculated by setting start and stop velocity thresholds. A start velocity of 0.10 m/sec and a stop velocity of 0.07 m/sec were used.

Anxiety scores

Both video recordings were used, without audio, to evaluate anxiety behavior. Anxiety was scored by the same person (observer), who was blinded to treatment day, to minimize variability and bias.

Anxiety scores for each dog's daily session (five sessions) were determined using a randomized blinded approach. Open field sessions for each dog were coded and scored in random order by a single observer. The videos were watched and assessed without sound so the observer remained blind to treatment day. Anxiety behavior was assessed and scored for each three minute time segment in the open field. These anxiety scores are subjective measures based on duration and intensity of anxiety behaviors observed over a given period of time. Scores were based on a scale of 1 through 6, where a score of 1 reflects no expression of anxiety behaviors, increasing stepwise

by half points, to 6 for severe anxiety behavior exhibited most of the time. The scoring rubric used was:

Anxiety	Definition
1	None; No anxiety for activity
2	Occasional and mild
3	Some of the time and mild / Occasional and moderate
4	Most of the time and mild / Some of the time and moderate / Occasional and severe
5	Some of the time and severe / Most of the time and moderate
6	Most of the time and severe

Scores were assigned in three categories of anxiety behavior for each 3 minute epoch, negative (passive), positive (active) and global (subjective intermediary of negative and positive scores). Negative (autonomic) anxiety behaviors included decreased activity, such as freezing, hiding, position against wall, or at door; lowered body postures, such as crouching, tail tucking and ears back; and autonomic /conflict behaviors, such as panting, shaking, salivating, yawning, lip licking, or elimination. Positive /increased anxiety behaviors included startling, bolting, vigilance, scanning, and active responses, such as pacing, aimless activity, stereotypic circling, retreat/escape attempts, digging, and climbing.

A calculated average (global mean) of the negative and positive anxiety scores was also calculated. Therefore, each dog had 4 scores for each 3 minute epoch (3 assigned by the observer and one calculated global mean) for a total of 12 anxiety scores for each 9 minute test session.

Results

Data from 16 dogs were included in the analyses. One dog ('Hunter') became destructive in the open-field arena on the first day of testing (control session 1). His open field test session on that day was terminated after 6 minutes; distance and anxiety scores truncated accordingly.

Motor activity

Table 9 shows the summary for the total distance traveled (meters). The data did not have equal variances (Levene's test; $p < 0.05$) so a natural log transformation was used in the analysis. Analysis of the transformed data showed sex ($p = 0.0298$) based differences, with males traveling more when compared with the female dogs (includes spayed females). Distance traveled by female dogs also decreased from the first to the last test; however, this effect was only marginally significant ($p = 0.0962$). Figure 11 shows the overall distance traveled by day for males and females.

Figure 11. Mean (± SEM) total distance traveled by day for females (Left) and males (Right).

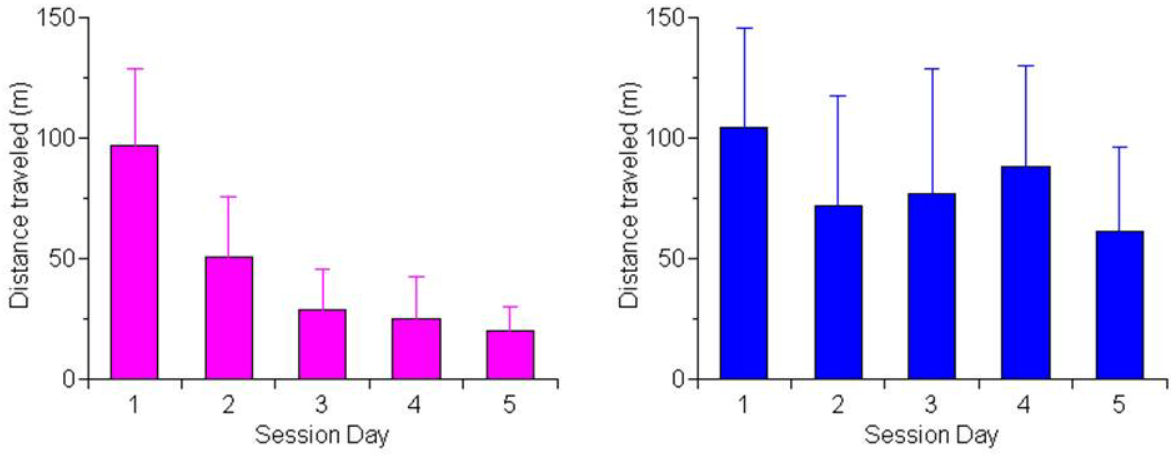


Table 10 shows individual dog data for each OFT session and endpoint analyzed. The following parameters were not affected by session day or sex: mean velocity ($p = 0.211$), door zone duration ($p = 0.0949$), and hidden zone duration ($p = 0.378$). A statistically marginal effect was seen for center-point/not moving duration ($p = 0.0520$). Mean (± SEM) values for days 1, 2, 3, 4, and 5 were 5.65 ± 0.63, 6.88 ± 0.58, 7.56 ± 0.55, 7.72 ± 0.51, and 7.71 ± 0.52 min, respectively. Post-hoc testing (Tukey's) was not statistically significant for this parameter. Statistically significant effects of sex ($p = 0.0019$) and session day ($p = 0.0355$) were seen for front wall zone duration (min).

Physiological parameters

Heart rate and rectal temperatures are presented for each dog in Table 11. A statistically significant difference in animal responses was seen between the first and second/third control sessions suggesting that the animals habituated to the open-field arena (data not shown). Data from the 2nd and 3rd control sessions were pooled in certain subsequent analyses. Mean heart rates and rectal temperatures are presented in Figures 12 and 13, respectively. There was no effect of sex or open-field test session on the change in body temperature seen during the open-field test. Heart rates in most dogs went down during the 9 minute open-field test. A statistically significant decrease occurred across the five open-field sessions ($p = 0.036$).

Figure 12. Mean (± SD) pre- and post- heart rates for the open field sessions for female (Top) and male (Bottom) dogs.

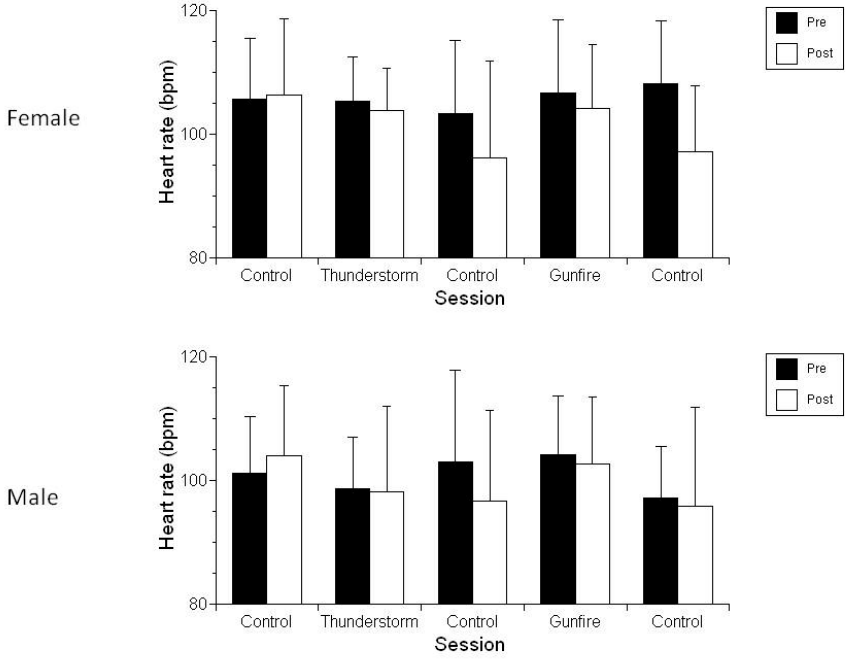
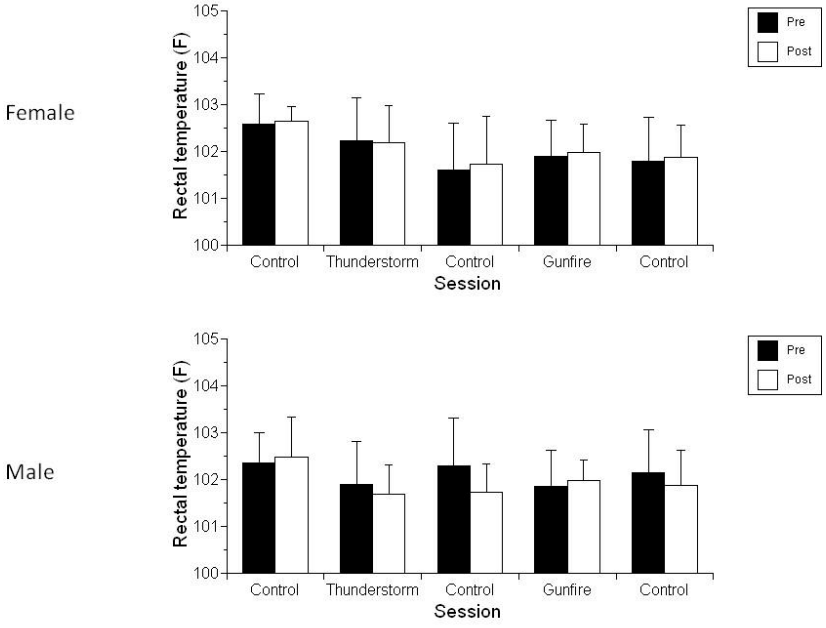
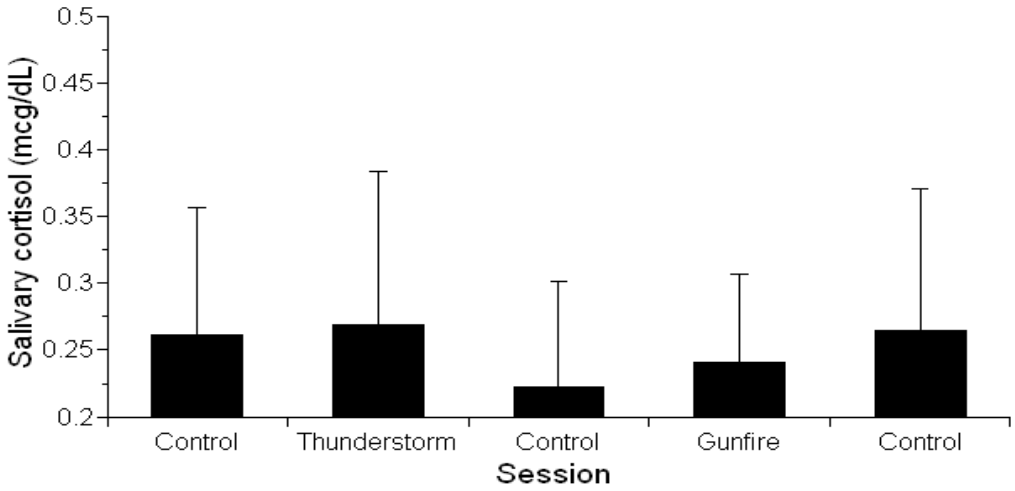


Figure 13. Mean (± SD) pre- and post- rectal temperature for the open field sessions for female (Top) and male (Bottom) dogs.



Mean (± SEM) salivary cortisol concentrations for days 1, 2, 3, 4, and 5 were 0.26 ± 0.02, 0.27 ± 0.03, 0.22 ± 0.02, 0.24 ± 0.02, and 0.26 ± 0.03 µg/dL, respectively. No effect was seen with respect to session day (p = 0.596 [Figure14, Table 12]).

Figure 14. Salivary cortisol concentrations by OFT day

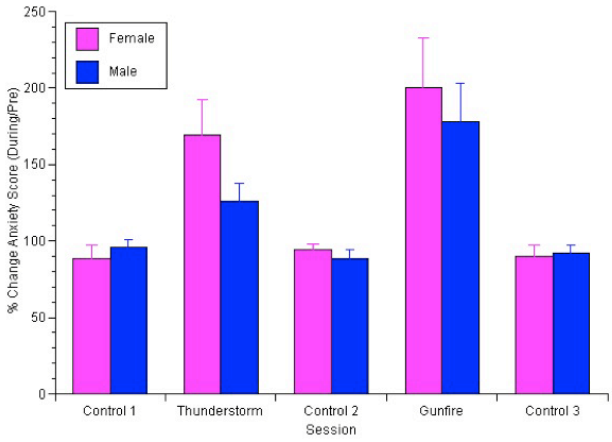


A statistically significant effect of sex was seen for this parameter (p = 0.0052). Mean (± SEM) saliva concentrations for male and female dogs were 0.28 ± 0.02 and 0.22 ± 0.01 µg/dL, respectively. However, this effect was not present when the data were analyzed as a % change from the day 1 concentration.

Anxiety scores

Table 13 shows the overall summary for this analysis while Table 14 provides individual dog data. Anxiety scores increased during both the gunfire and thunderstorm sessions. Figure 15 shows the data when normalized for the % change in anxiety score. The normalization procedure accounts for a change in anxiety scores between the first three minutes (pre) and the second three minutes (during), when the adverse stimulus (or not) occurs. Anxiety scores did not go up significantly between control sessions 1, 2, or 3 but do go up with both the gunfire and thunderstorm sessions. Analysis of this data shows an overall effect - but there is no statistically significant difference (p = 0.0948) between either the thunderstorm or gunfire sessions. Subsequent analyses pooled this data and the data for male and female dogs (p = 0.1783). Figure 8 shows the linear correlation between NCSU ERT anxiety score (see Phase I) and the % change in the open field anxiety score (100% means no change). If we include Piper the p value was 0.0299 with an r^2 value of only 0.294. If we exclude Piper then the p value was 0.0005 with an r^2 value of 0.62 (Figure 7).

Figure 15. Mean (± SEM) normalized anxiety scores for male and female dogs during the open field test.



Based on the results of the open field anxiety scores, dogs were able to be categorized into two groups: those that had the greatest change in anxiety score during treatment periods ("worst" dogs), and those with a smaller change in anxiety score during treatment periods ("non-worst" dogs). Dogs in the "worst" group had changes in mean global anxiety scores greater than 1 (see Table 15). Differences between these two groups (worst and non-worst) on the ERT were examined and discussed in Phase I.

Additional statistical analyses

Total distance traveled during the 9-minute open field test session was not correlated with the dog's normalized anxiety score during the middle 3 minute portion of the test ($p = 0.172$, data not shown). There was a statistically significant association between the dog's normalized anxiety score and center-point /not moving duration; however the strength of the association was very weak ($r^2 < 0.05$). We found that the change in heart rate (pre – post) was not correlated with the total distance traveled during the test session ($p = 0.4793$, data not shown). Likewise, the change in heart rate (pre – post) was not correlated with the dog's normalized anxiety score ($p = 0.1912$, data not shown). The change in heart rate was however, correlated with the dog's sex ($p= 0.0488$). Female dogs demonstrated a greater change in heart rate in response to an auditory stimulus when compared to male dogs (data not shown).

Discussion

As predicted, we observed an increased expression of behaviors associated with fear and anxiety in dogs during the open field audio thunderstorm and simulated gun battle sessions, compared to control periods. In this experiment there was no significant difference between the dogs' behavioral responses to playback of the sound of either a thunderstorm or gun fire. The subjective magnitude of the anxiety response was moderate and varied among dogs. We found a positive and statistically significant correlation between the NCSU-rated emotional reactivity test (ERT) scores and anxious behaviors in dogs during the open field thunderstorm and simulated gun battle sessions. Interestingly, the sound intensity used for the playback of the sound of either a thunderstorm or gun fire was qualitatively equivalent and resulted in similar behavioral and physiological responses. Our finding further validates the utility of the ERT in predicting certain behaviors in dogs. In addition, on the basis of the mean global open field anxiety score, we were able to categorize dogs into two groups, "worst" and "non-worst," which allowed us to evaluate

the ERT responses of the "worst" dogs to provide predictive validity of the ERT test in the selection of candidate IDDs (see Phase I report).

We also observed habituation responses that occurred within and between open field sessions. For example, the dog's activity (as assessed by total distance traveled) was highest during the first control (no sound stimuli) session. Indeed, activity was also highest in the beginning of the test session (especially in female dogs), then decreased over sessions. An adaptive central nervous system phenomenon, habituation is the decreased response to a continuous or repeated stimulus over time. In the open field, habituation was a normal response as the dog became familiar with the open field environment within days and over days. Deviations from the observed pattern of habituation represent a behavioral response.

Although we detected measurable behavioral responses to provocative sound stimuli, we detected minimal changes in physiological measures of stress (e.g., heart rate and salivary cortisol concentration). The lack of a more robust physiological effect likely reflects the strength and duration of the stress stimuli used (3 minutes), and the fact that the physiological measurements were taken after the post-stimulus period rather than during the stimulus. A measurement refinement would be to collect heart rate data in real time during stimulus presentation. In addition, since our post-ERT plasma cortisol measures were a more sensitive measure of stress response than post-ERT salivary cortisol measure, collection of the plasma cortisol after sound stimuli might reveal a stress response.

The magnitude of the behavioral response for any individual is influenced by the sound intensity (Overall, 2002). Thus, a more robust sound stimulus might produce a more profound behavioral response. For example, response to a low intensity sound stimulus might include pacing, panting, or staying close to a handler. Response to a more intense sound stimulus might result in more extreme response, including attempts to hide or flee from the sound source or freezing for extended periods of time (Branson and Rogers, 2006; Voith and Borchelt, 1985).

In conclusion, our open field model produced a measurable anxiety/stress behavioral response in dogs and provided validation for the NCSU USMC ERT tests. This model may be used in future experiments to examine mitigation strategies in candidate IDDs. Our research also suggests that a three day open field test, perhaps using more intense stimuli, could be a useful adjunct to the ERT in the selection of candidate IDDs.

PHASE III. OBJECT DISCRIMINATION

Background

Performance as an improvised explosive detector dog (IDD) requires the animal to learn to execute actions the dog would not normally perform and, moreover, to execute those actions under human command. For example, the dog must learn to assume sternal recumbency ("cover") when it detects a specific odor, rather than investigate the odor ("aggress"). Trainability is therefore an important factor to consider in the selection of candidate IDDs. Trainability is related to a number of factors, including intelligence. Intelligence, in turn, has a variety of different dimensions or domains. Examples of different cognitive domains in dogs can include instinctive intelligence (e.g., retriever or herding skills), adaptive intelligence or problem-solving ability, and working and obedience intelligence.

The ability of a dog to function in the role of an IDD is likely linked to different cognitive features, such as self-control, motor-control, signal processing, and intelligence (Helton, 2007). There has been a considerable increase in the number of studies on dogs' cognitive abilities (Marshall-Pescini et al., 2009). One focus of these studies is to determine whether there are differences in trainability amongst breeds or breed groups (Ley et al., 2009; Serpell and Hsu, 2005). Since the USMC uses Labrador Retrievers as IDD, the issue for our study is not inter-breed performance, but rather differences in cognitive performance among individual members of this group (intra-breed performance).

The goal of this phase of the project is to evaluate cognitive performance of Labrador Retrievers on a visual discrimination and object reversal task. Discrimination learning refers to a paradigm in which a subject is allowed to respond to one of at least two alternative stimuli, one of which is deemed to be correct. If the subject responds to the correct stimulus, it receives a small food reward (Milgram, 2003). Performance on this task assesses learning and memory, impulse inhibition, and several other cognitive domains of importance for candidate IDDs. Questions that our research attempts to answer is whether sex, coat color, or emotional resilience influence cognition in Labrador Retrievers. This information can then be used to improve selection criteria for candidate IDDs.

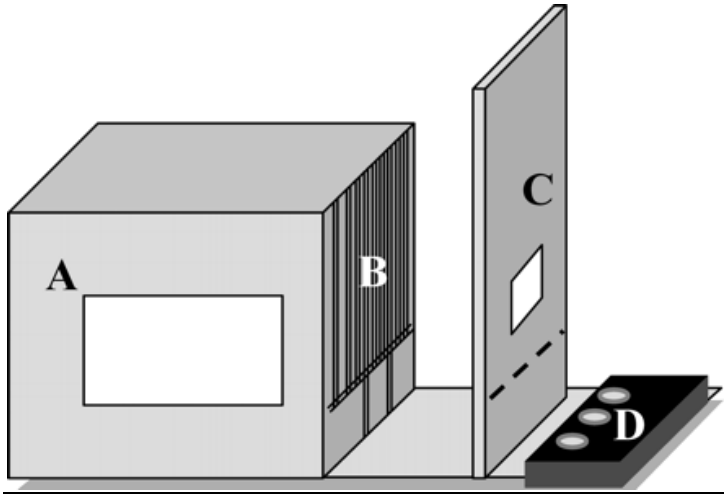
Materials and Methods

Test apparatus

The testing apparatus (CanCog, Toronto) was a 66 cm x 178 cm x 91 cm plastic chamber based on a Toronto General Testing Apparatus for canines (Figure 16). The chamber was positioned on a cart approximately 64 cm from the ground, equipped with a ramp measuring 114 cm x 58 cm connecting to the rear of the chamber for access. Stainless steel bars separated the portion in which the dog remained (measuring 66 cm x 127 cm x 91 cm) from the area where stimuli were presented (measuring 66 cm x 46 cm x 91 cm). The experimenter sat in front of the testing chamber, visually separated from the dog by a one-way mirror. The front wall of the chamber contained a hinged door measuring 20 cm high. A black, sliding Plexiglas tray with three wells was used to present stimuli by sliding the tray into the chamber through the hinged door. The stainless steel bars could be adjusted for each dog to create three openings allowing the dog to obtain food rewards from the wells. A small food treat (approximately 2 cm of Pup-Peroni™ Original bacon flavor treats) was used as the reward. The chamber and equipment were wiped with a dry cleaning towel in between subjects. After all subjects completed testing for the day, the

chamber was cleaned with disinfectant solution (47 ml Virkon powder dissolved in 590 ml of water).

Figure 16. Schematic of the testing apparatus used in cognitive assessment testing (from Araujo and Milgram, 2004). **A**. The test box where the dogs entered from a ramp. **B**. The front of the test box that consisted of stainless steel bars of adjustable height that provided three openings to access the objects. **C**. A plastic screen between the experimenter and animal. The plastic screen had a one-way mirror and a hinged door, which was lowered to present the sliding tray with objects to the animals. **D**. A black Plexiglas presentation tray that had three food wells, two lateral and one medial.



Data was collected using DogCog software (CanCog Technologies, Toronto, Canada) on a computer running Windows 7 interface. The software was used to randomize the location of the rewarded well on each trial, signal the start of a trial with a tone, and time response latencies and inter-trial intervals. A key stroke recorded the dogs' choice on each trial.

Stimuli presentation

Stimuli used were plastic, three-dimensional solid colored shapes of various colors and sizes. The pre-training phase employed 6 cm x 6 cm x 7.5 cm solid white blocks and 21 unique solid colored shapes for the object-approach phase. The visual discrimination and reversal phases used a 4-pronged blue rectangular shaped block measuring 12.5 cm x 5 cm x 6 cm and a one-pronged yellow 6 cm cube. Objects were mounted onto white plastic coasters measuring 10 cm in diameter.

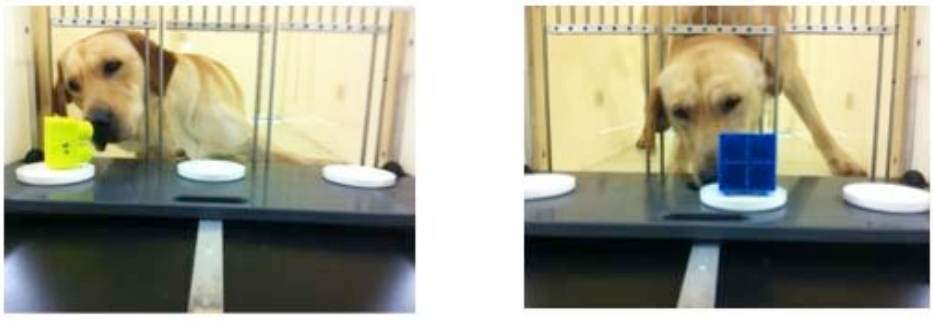
Test procedures

Pre-training: A five-phase pre-training procedure adapted from CanCog™ was used to habituate the dogs to the testing apparatus and procedure. First dogs were acclimated to the chamber and movement of the gate and presentation tray while learning to eat food rewards from the tray. Next, dogs were trained to approach the presentation tray and readily consume food rewards from all 3 wells. Then, only one well was rewarded at a time and dogs were trained to inspect the wells before approaching while using the corresponding gate for each well. Next, objects (white plastic blocks attached to white plastic coasters using Velcro) were placed over the wells and dogs were trained to displace all three blocks using their nose to access a covered food well (Figure 17). Finally, dogs were trained to displace a single object (colored blocks) to obtain a food reward. Only one object was presented at a time, with 21 different objects used in the session.

Pre-training phases included:

a. Adaptation. Acclimate all dogs to the CanCog system. A food reward is provided in all wells.
b. Phase 1. Dogs are trained to use the proper gate (right, center, left). 10 trials with a reward in each well.
c. Phase 2. A 21-trial session with a 30-second inter-trial intervals Only one well is rewarded on each trial. 2-second "inspection interval" before a choice is made. Criterion = 16/21 correct responses
d. Phase 3: Dog learns to displace objects. 15 trials with a reward in each well. Wells covered with a white block.
e. Phase 4: 21-trial session. One well is rewarded and covered with a coaster with an object attached. 21 different objects are used. Criterion = 16/21 correct responses.

Figure 17. Dog using the proper gates in the CanCog system to successfully manipulate objects with its nose.



Object discrimination learning: A preference test was conducted to determine which of two objects, a yellow square block or a blue rectangle, would serve as the rewarded (positive) object and which would serve as the unrewarded (negative) object. Both objects were simultaneously presented for 10 trials, with both wells rewarded on each trial. Both objects appeared an equal number of times in the left and right positions in randomized order. The object in which the dog responded to more than 5 times during the preference test was designated as the positive stimulus and the other object as the negative stimulus for the subsequent object discrimination learning phase. If the dog did not make a choice preference then a coin flip was used to assign a positive stimulus.

Object discrimination sessions were conducted daily and consisted of 20 trials. A tone signaled the beginning of a trial at which point the hinged door was lowered and the stimulus presentation tray was inserted halfway into the inspection chamber for a 3 s inspection interval. The tray was then fully inserted into the inspection chamber and the dog was allowed 30 s to make a response. A response to the rewarded object (S+) revealed a food reward in the well. Responses to the unrewarded object (S-) were not rewarded and a correction procedure allowed the dog to then respond to the correct object and obtain the food reward. Responses were recorded and then next trial began following a 30 s inter-trial interval (ITI). If a response was not made within the 30 s time allotment, the tray was withdrawn, a non-response was recorded and the next trial began. A food reward was fixed underneath the negative stimulus object to control for odor cues. Object position throughout the session was randomized and balanced such that each object was presented on the left and right side an equal number of times, with no more than 3 trials in a row containing the S+ on the same side. Only the left and right wells were used during this phase.

Criterion was met when the subject responded correctly on 16/20 trials or better on one session, followed by a total of 28/40 correct responses over two consecutive sessions.

Reversal learning: After acquiring the initial object discrimination task, the S+ and S- were switched to test for reversal learning. The procedure was the same as the previous phase except that the object previously designated as the S+ became the S- and vice versa.

Data analysis

For each task, error scores were calculated by summing the total number of errors committed up to and including the last criterion day. A multivariate ANOVA was used to determine whether sex or coat color were significantly associated with cognitive test scores. A multivariate ANOVA was also used to determine whether the NCSU ERT score or anxiety in the open field were significantly associated with cognitive test scores. The dog's anxiety in the open field was highest during the sessions where playback of recorded thunderstorm or simulated gunfire sounds was played (see Phase II for additional details). Mean normalized anxiety scores (see Phase II) for each dog were calculated from their individual thunderstorm and gunfire sessions and used in this analysis. We also evaluated whether dog performance on either task was associated with their categorical designation as "Worst" or "Non-worst" on the open field anxiety test.

Results

All dogs were able to complete all pre-training phases (Table 16). The mean number of trials (± SEM) required to complete the training was 9.6 ± 0.6. One dog ('Honey') required 9 sessions to become comfortable with the CanCog unit. This response was consistent with her low emotional resilience seen during our previous ERT evaluation (see Phase I). This response, however, was not seen in other dogs with similar low ERT scores. The total number of trials required to complete pre-training was not affected by sex, coat color, NCSU ERT score, or OFT anxiety score. We also examined the effect of age and coat color on the number of trials required to reach criteria for the reward approach learning task. Coat color, but not sex, had a marginally statistically significant ($p = 0.0565$, Pearson's χ^2) effect on the number of reward approach learning trials needed to reach criterion. The mean (± SEM) number of reward approach learning trials needed to reach criterion were 3.1 ± 0.4 and 1.6 ± 0.4 for black- and yellow-coated dogs, respectively.

All dogs completed the object discrimination task and 15/16 completed the reversal task. The one dog ('Piper') that did not complete the reversal task also required more trials to complete the

object discrimination task. Her low performance was thought to reflect low motivation for the food reward. Data from this dog was deleted from future analysis. As mentioned in Part I, one dog ('Reno') had a single isolated seizure episode during the course of the study. During our statistical analyses we evaluated the impact of including or excluding this dog from the analysis. Our conclusions reached for the object discrimination and reversal tasks remain the same irrespective of whether this dog's data were analyzed. Therefore, the data presented includes this animal.

Table 14 presents the individual animal data for the object discrimination and reversal tasks. Figure 18 shows a graphical representation of the performance of two dogs during the object discrimination and reversal tasks. Dogs had more difficulties on reversal learning than on original learning, as indicated by differences between object discrimination reversal and object discrimination learning. The mean (± SEM) error rates on the discrimination and reversal tasks were 21.6 ± 1.3 and 44.9 ± 1.8%, respectively. Dogs also required more trials to acquire the reversal task. The mean (± SEM) number of trials needed to reach criterion on the discrimination and reversal tasks were 117 ± 6.6 and 197 ± 14.3, respectively. There were no overall sex- or coat color- effects on visual object discrimination learning (trial number or error rate). Sex, but not coat color, had a statistically significant effect on the number of trials needed to reach criterion on the reversal task ($p = 0.019$). The mean (± SEM) number of reversal task trials was 231.4 ± 21.8 and 167.5 ± 11.9 for female and male dogs, respectively. Coat color, but not sex, had a statistically significant effect on the reversal task error rate ($p = 0.0297$). The mean (± SEM) error rates on the reversal task were 47.6 ± 1.7 and 39.6 ± 3.1% for black- and yellow-coated dogs, respectively. Dogs rated as "Worst" on the open field anxiety test required more trials (234.3 ± 22.1) to master the reversal task than did dogs rated "Non-worst" on this task (165.0 ± 9.1, $p = 0.0094$).

The ERT score was linearly correlated with the number of trials to reach criteria for the visual discrimination ($p = 0.0457$, $r^2 = 0.27$) and reversal task ($p = 0.0457$, $r^2 = 0.27$) (Figure 19).

Figure 18. Performance of two dogs ('Bullet' and 'Annie') during an object discrimination reversal task. [Note: In this task the dog, having learned to respond to one of two simultaneously presented objects in order to obtain reward (data presented in the left panel of each figure), is required to inhibit the response towards the rewarded (S+) object, and instead, to begin responding to the other previously unrewarded object, in order to gain reward (data presented in the right panel of each figure)].

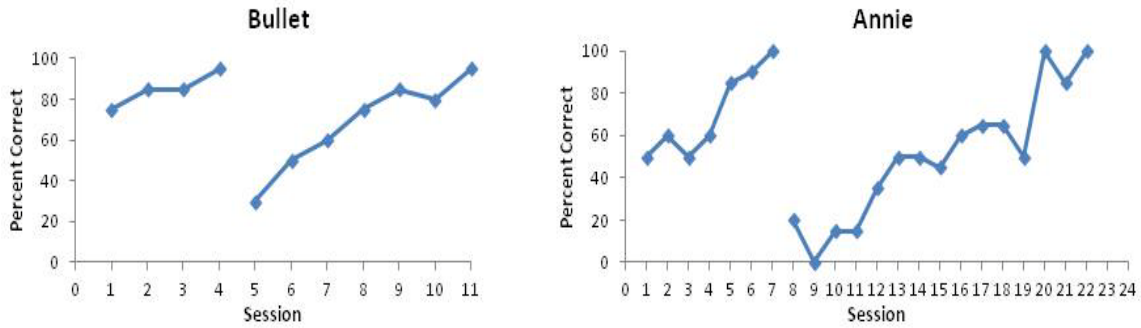
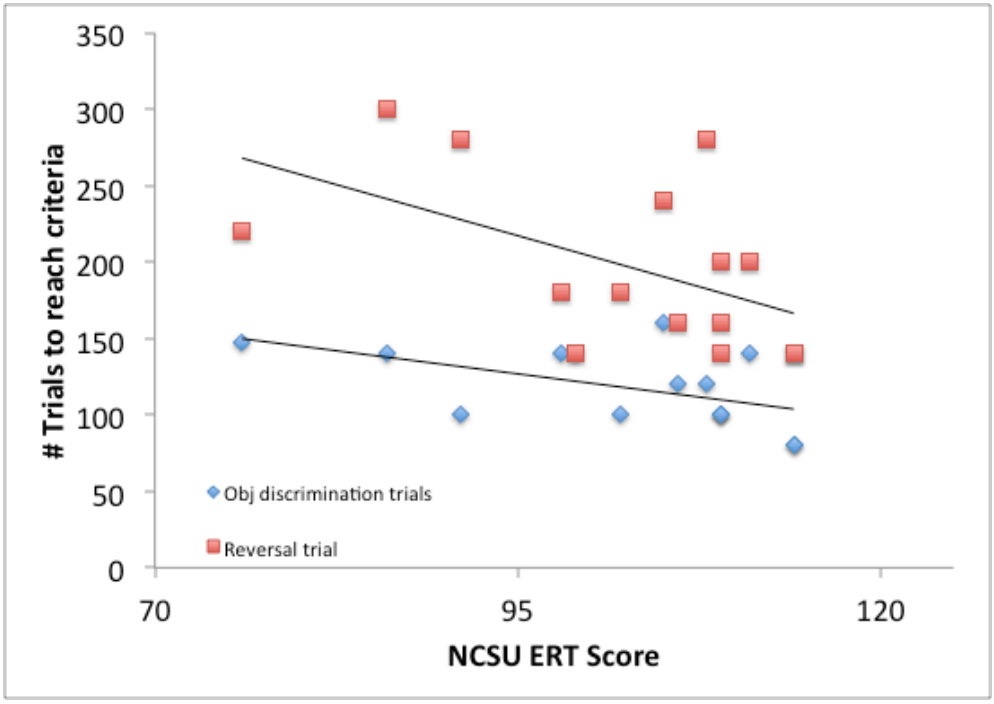


Figure 19. Linear correlations seen between NCSU ERT scores and the number of trials to acquire the object discrimination and reversal tasks.



Discussion

The system designed by CanCog™ has been used by other investigators to evaluate cognitive function in dogs following aging, dietary manipulation, and pharmaceutical administration (Araujo and Milgram, 2004; Callahan et al., 2000; Chan et al., 2005; Milgram 2003; Studzinski et al., 2005). These and many other experiments have shown that object discrimination and reversal learning are influenced by function of age, task difficulty, pre-existing object preferences, and other factors. Performance on this test can also be influenced by a variety of non-cognitive factors (e.g., satiety). Odor cues related to the food reward were minimized by having food associated (but unavailable) with the unrewarded (S-) stimulus.

We found that Labrador Retrievers performed poorer on the reversal task when compared to their ability to learn the initial object discrimination test. This finding likely reflects the increased difficulty of the reversal task. Our findings are consistent with other reports that dogs show slower learning on reversal than on original discrimination tasks (Boutet et al., 2005). Discrimination reversals require subjects to inhibit responses to previously correct stimuli and to shift responses to a new stimulus-reward contingency within the same perceptual dimension (Tapp et al., 2003). The relatively poor performance of dogs in reversal tasks has been attributed to perseverative responding - uncontrollable repetition of a particular response (Boutet et al., 2005). The ability to eventually overcome perseverative responding and adapt to changes in stimulus-reward contingencies is related to general learning ability, with quicker reversal learning indicative of faster general learning and adaptability.

One of the first goals of the present study was to further characterize the effects of sex and coat color on cognitive function in the dog. Female dogs required more trials (approximately 38% more) to acquire the reversal task when compared with male dogs. Sex has been shown to influence responsiveness to environmental stimuli, to correlate with levels of interest in novel items, and to define the extent of participation in activities in nonhuman primates and ravens (Fragaszy and Visalberghi, 1990; Range et al., 1996). Boys and juvenile male monkeys perform better on simple visual discrimination task than their female counterparts (Overman et al., 1996). This response was not however, seen in infant macaque monkeys tested on a two-object visual discrimination task (Ha et al., 2011).

Coat color also influenced cognition with black-coated Labrador Retrievers. Black-coated Labrador Retrievers performed poorer on the reversal task, and had an approximately 20% higher error rate on the reversal task when compared with yellow-coated dogs . Black-coated Labrador Retrievers also performed poorer on the reward approach learning task. This task was intended to teach subjects to use visual cues in order to locate a reward. Subjects were trained daily until they immediately displaced the object at criterion levels. Errors on these tests could be caused by motivational factors, memory errors, lack of flexibility in responding, and incorrect associations with reward. Error factors can include stimulus-perseveration, differential cue, response shift, and position-habit errors. The biological basis for this difference in performance bwteen black- and yellow-coated retrievers is unknown.

Reward- and object-approach learning in dogs depend on procedural learning (Head et al., 1998). Milgram and coworkers (1994) previously reported that age sensitivity of these two tasks varied as a function of breed and/or source of the dog. Since we used a single breed this is unlikely to be a factor. However, the source of the dog may reflect differences in previous experience. Prior odor imprinting training at K2 did not affect reward and object approach learning ($p = 0.181$). As previously reported, dogs show significant age-dependent deficits on an object reversal learning task (Milgram et al., 1994; Tapp et al., 2003). In our study, an age-dependent effect on reversal

learning was not observed. This finding is not unexpected since the range of ages in the examined cohort of dogs was narrow, while other investigators compared juvenile to senescent animals. However, we cannot rule out that other factors related to the dog's previous experience influenced the results of our study.

The second goal of this study was to characterize the relationship between cognitive function and emotional resilience. Emotional resilience was quantified using the NCSU emotional reactivity test (see Phase I) and the anxiety phenotype ("Worst" or "Non-worst") that was established using the open field anxiety test (see Phase II). We observed that some individual dogs demonstrated anxious behaviors in the CanCog test system and required more pre-training acclimation trials than other animals. Reduced emotional resilience (as measured using the NCSU ERT) was associated with decreased cognitive performance in both tasks (as determined using number of trials). Although statistically significant differences were seen the magnitude of the response was small ($r^3 < 0.3$). This result likely reflects the low stress test environment used for this investigation (e.g., quiet testing room, single technician performed all assessments, etc). We also observed that dogs with an anxiety phenotype ("Worst) required more trials to acquire the reversal task. We predict that dogs with lower emotional resilience and/or an anxiety phenotype would have more difficulty learning new tasks under more stressful conditions (e.g., battlefield environment) than dogs with the more robust behavioral phenotype. In further studies, the link between behavioral characteristics, physiological parameters, and learning abilities in dogs should be further investigated to reveal whether the relationships of explorative behavior and learning abilities reflects personality traits, or if slow learners are simply less motivated to learn, but more motivated to play. Work performed in subsequent phases was designed in part to answer this question.

PHASE IV. DELAYED NON MATCH TO POSITION (DNMP)

Background

The ability to learn about locations and orientations of objects in the environment is critical to an animal's ability to survive and reproduce. Memory for locations of previously visited sites containing food sources, mates, or predators requires sophisticated spatial cognition and memory in which a number of learning strategies may be adapted, including the use of visual landmarks (Fiset, 2007; MacPherson and Roberts, 2010). These types of abilities rely on learning about locations of objects and relationships between them in space – a process known as spatial memory (Johnson and Adamo-Villani, 2010). Previous research has found implications for spatial learning and memory in the basic processes involved in search behavior in dogs (Fiset, 2000), which may be an important consideration in improvised explosive detector dog (IDD) trainability and performance.

A component of spatial memory includes working memory, the memory system involved in continuously storing and updating current information before being stored to relatively permanent memory. The delayed non-matching to sample (DNMS) procedure is commonly used to assess working memory in humans and animals, involving the presentation of one object (the sample), followed by a delay and then a choice between the same object and a new one. Choosing the novel object results in a reward, requiring a memory for which object was presented as the sample. A spatial version of this task, known as delayed non-matching to position (DNMP), involves presenting identical objects in various locations. During the sample phase, one object is presented in a given location. Following a delay, the object appears again in the same position, with an identical object in a novel location. Choosing the object in the novel location is rewarded, requiring memory for where the object had appeared prior to the delay. The DNMP task has been a useful tool in assessing spatial memory learning and delay in beagle dogs (Chan et al., 2002).

The goal of this phase of the project was to evaluate cognitive performance of Labrador Retrievers on a spatial learning and memory task. Performance on the DNMP task assesses a variety of cognitive domains and may be predictive of performance important for candidate IDDs. Questions that our research attempts to answer are whether sex, coat color, or emotional resilience effect spatial memory learning in Labrador Retrievers. This information can then be used to improve selection criteria for candidate IDDs.

Materials and Methods

Test apparatus

The testing apparatus was a 66 cm x 178 cm x 91 cm plastic chamber based on a Toronto General Testing Apparatus for canines (Figure 16; See Phase III). The chamber was positioned on a cart approximately 64 cm from the ground, equipped with a ramp measuring 114 cm x 58 cm connecting to the rear of the chamber for access. Stainless steel bars separated the portion in which the dog remained (measuring 66 cm x 127 cm x 91 cm) from the area where stimuli were presented (measuring 66 cm x 46 cm x 91 cm). The experimenter sat in front of the testing chamber, visually separated from the dog by a one-way mirror. The front wall of the chamber contained a hinged door measuring 20 cm high. A black, sliding Plexiglas tray with three wells was used to present stimuli by sliding the tray into the chamber through the hinged door. The

stainless steel bars could be adjusted for each dog to create three openings allowing the dog to obtain food rewards from the wells. Approximately 2 cm in of Pup-Peroni™ Original bacon flavor treats were used as rewards. The chamber and equipment were wiped with a dry cleaning towel in between subjects. After all subjects completed testing for the day, the chamber was cleaned with neutral table cleaner disinfectant solution (1.6 oz Virkon powder dissolved in 20 oz of water).

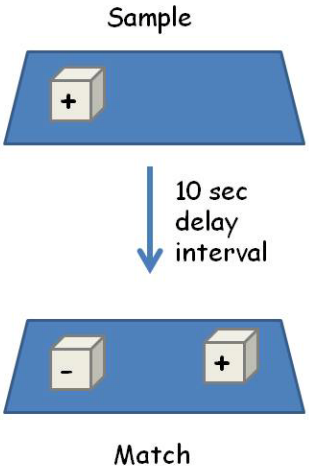
Data was collected using DogCog software on a computer running Windows 7 interface. The software was used to randomize the location of the rewarded well on each trial, signal the start of a trial with a tone, and timed response latencies and inter-trial intervals. A keystroke recorded the dogs' choice on each trial.

Test procedures

All dogs underwent a 5 phase pre-training procedure aimed to acclimate subjects to the testing apparatus and procedures and an initial visual object discrimination task (see Phase III) prior to beginning the DNMP phase.

A trial began with the signal of a tone and the presentation of a single object (white block) serving as the sample in one of the three positions. Following a 2-s inspection interval the tray was fully inserted and responses to the object were rewarded. The tray was then removed and a 10-s delay was simultaneously initiated. Following the delay the tray was again presented with two identical white blocks, one in the original sample position and another in one of the other two positions. After a 2-s inspection interval, a second tone sounded and the tray was fully presented. Responses to the object in the position different than the sample were rewarded (S+). A correction procedure, used only on the first error of the session, allowed the dog to continue to respond after an incorrect response (S-) until the food reward was obtained. The remainder of the trials in the session ended after the first response, regardless of whether it was correct or incorrect. The tray was then removed and a 30-s inter-trial interval began until the next trial began. The blocks were wiped in between trials and the block used as the sample as well as the S+ and S- positions, were interchanged throughout the session to control for scent marking of the objects. A food reward was placed under the S- object to control for odor detection. DNMP sessions consisted of 12 trials in a session, with each position (center, left, and right) serving as the S+ an equal number of times. A two-part criterion was used for this phase. The first part of the criterion required either 11/12 correct responses or better on one session, 10/12 or better on 2 consecutive sessions, or 3 consecutive sessions of 10/12, 9/12, 10/12, in that order. The second part of the criterion required 70% correct or greater total over 3 consecutive sessions. Figure 20 provides a schematic representation of the test.

Figure 20. Delayed non-matching to position (DNMP) paradigm. Modified from Adams et al., 2000.



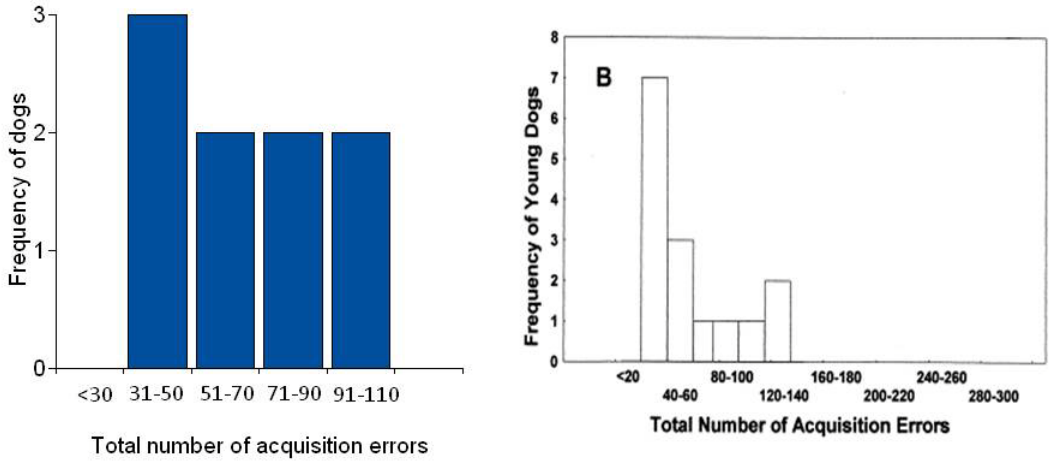
Data analysis

For each task, summing the total number of errors committed up to and including criterion day was used to calculate error scores. A multivariate ANOVA was used to determine whether sex or coat color was significantly associated with cognitive test scores. A multivariate ANOVA was also used to determine whether the NCSU ERT score or anxiety phenotype ("worst" or "non-worst") in the open field was significantly associated with cognitive test scores. We also examined whether performance on the object discrimination (Phase III), object reversal (Phase III), and DNMP tasks were predictive of one another. One dog ('Reno') had a single isolated seizure episode during the course of the study. During our statistical analyses we evaluated the impact of including or excluding this animal from the analysis. For some analyses, the number of trials was right censored at 300 with the error rate calculated accordingly. Response accuracy has proven to be a powerful behavioral indicator of learning and memory performance in dogs tested in the CanCog™ system (Callahan et al., 2000; Chan et al., 2005, Head et al., 1998, Milgram et al., 1994, Milgram et al., 2003; Tapp et al., 2003).

Results

All dogs were able to complete all pre-training phases (Table 15). One dog ('Piper') did not successfully complete acquisition of the object discrimination and reversal tasks (Phase III). Because of her low motivation she was not tested on the DNMP task. Six additional dogs ('Annie', 'Bullet', 'Dakota', 'Jimmy', 'Reno', and 'Rip') did not complete the DNMP task (terminated at 300 trials, 25 days of training). Individual results for the DNMP task are presented in Table 17. The dogs in our cohort segregated into two DNMP performance categories: (a) fast learners, and (b) slow learners (acquisition did not occur before 300 trials). Among the fast learning dogs that acquired this task (n = 9), the mean (± SEM) number of trials to acquire the task was 187.8 ± 19.2 trials. The mean (± SEM) error rates in the fast and slow DNMP learners was 34.9 ± 1.0 and 45.7 ± 2.7%, respectively. A frequency histograms showing the total number of acquisition errors at a 10 sec delay is presented in Figure 21. Data for young beagle dogs from Adams et al (2000) is also presented in this figure.

Figure 21. Frequency histograms showing the total number of errors for Labrador Retrievers to reach criterion at a 10 sec delay (Left). Only includes data from dogs that completed acquisition of the DNMP task. Data for young beagles (Right) with a similar DNMP task (10 to 30 sec delay) is shown for comparison (Adams et al., 2000).



We also examined the effect of sex and coat color on the number of trials required to reach criteria for the DNMP test. Neither sex nor coat color had a statistically significant effect on the number of trials needed to reach criterion or the associated overall error rate. Likewise, the classification of the dog's anxiety phenotype ("Worst" or "Non-worst") and ERT scores were not associated with either of these metrics of DNMP performance. These conclusions were true for all dogs that attempted the DNMP task or acquired the task. Likewise, we did not see a correlation with either the number of trials needed to reach criteria on the DNMP with trial number to criteria on the object discrimination or reversal tasks. The same was also true for DNMP error rate.

Discussion

The DNMP has been used to evaluate several diverse aspects of spatial learning and memory in young and old dogs (Adams et al., 2000). These include acquisition (i.e. spatial learning), spatial working memory (i.e. the process of maintaining a limited amount of information in an active representation for a short period of time so that it is available for use), and maximum spatial working memory capacity. In this task, animals are rewarded if they respond to an object located on the side opposite to the location of the object presented on the preceding sample presentation. Head and coworkers (1995) have reported that a higher proportion of aged dogs could not acquire the DNMP task relative to young dogs and the acquisition rate was correlated with age.

The dogs in our cohort segregated into two DNMP performance categories: (a) fast learners, and (b) slow learners (acquisition did not occur before 300 trials). The mean (± SEM) error rates in the fast and slow DNMP learners was 34.9 ± 1.0 and 45.7 ± 2.7%, respectively. The rate of acquisition of the DNMP test did not correlate with performance on our previous cognitive tests. This is not unexpected since the cognitive domains used to discriminate objects differ from neural substrates required for spatial learning and working memory.

There is still much debate over sex differences in spatial working memory in animals (Jonasson, 2005). In rodent working memory tasks involving the retention of largely spatial information,

such as the Morris water and the radial-arm maze procedures, males typically outperform females (Jonasson, 2005). There are several published rat studies using the same procedure employed in our dogs that also assessed the behavior of both male and female rats (Aarde and Jentsch, 2006; Marrs et al., 2005). In these studies, female rats performed better than males. However, in other tests of spatial working memory, there are either no differences between the sexes or better performance by females (Aarde and Jentsch, 2006). Importantly, neither sex nor coat color was a determinant for performance in our cohort of Labrador Retrievers.

Anxiety phenotype or NCSU ERT score did not predict performance on the DNMP test. This was unlikely associated with our decision to curtail training on the DNMP test after 20 test days (300 trials) since an analysis of simulated data involving a larger number of trials (i.e., 400 trials) did not reveal an effect.

In conclusion, this study demonstrates that spatial learning and working memory in Labrador Retrievers is variable. Dogs can be segregated into two populations with different learning rates. Other tests of emotional resilience and cognition (object discrimination) are not predictive of this capability thus the development of alternative short-term screening tests (see Phase VI) is desirable.

PHASE V. OLFACTORY DISCRIMINATION

Background

The performance of an improvised explosive device detecting dog (IDD) relies fundamentally on its ability to detect odor. Successful IDD performance is related to general learning and memory processes as well as olfactory sensitivity. A suitable candidate IDD must not only be adept at odor discrimination, but must also possess trainability and willingness to learn and remember a variety of odors. How rapidly a candidate dog learns to discriminate odors may be indicative of its potential for success as an IDD.

Although canines possess a remarkable capacity to recognize a variety of odorants, little is known about the underlying processes of this process, such as how dogs learn to detect odors what variables influence their effectiveness, or how to enhance their functional abilities (Williams and Johnston, 2002). Further, most research regarding canine olfactory learning and sensitivity has been anecdotal or based on field studies with multiple variables. A more methodical approach to better understand the capabilities and challenges involved in IDD performance is needed. Laboratory studies of olfactory discrimination allow for a controlled analysis of olfactory learning and sensitivity in which a number of variables may be investigated including olfactory thresholds, rate of acquisition of learned response, and generalization to chemically-related odors (Harper et al., 2005; Williams and Johnston, 2002).

An important question with regard to olfactory thresholds is which characteristics of a multi-component odor are responsible for positive detection by an IDD? Canines may signal either to the parent explosive, to non-explosive chemical markers associated with the explosive, or both. Identification of key chemicals and the emanating odors that elicit detection would lead to more efficient training methods by requiring fewer training substances (Harper et al. 2005). It is thought that dogs typically learn to respond to the most abundant chemical odor of a target explosive (Johnston, 1999). In fact, dogs may alert to a single “dominant” odor to which they have been trained. Training methods may be optimized by training dogs on low quantities of single key chemicals (Harper et al., 2005). A related consideration, which has received little investigation, is whether or not dogs trained on a single target odor will generalize to other untrained odors of chemical similarity. Identification of target substances that produce generalization to related compounds could greatly improve training efficiency (Johnston, 1999).

The goal of this phase of the project was to evaluate performance of Labrador Retrievers on an olfactory discrimination task and to establish a laboratory protocol that may be used to investigate the questions elucidated, above. Discrimination learning refers to a paradigm in which a subject is allowed to respond to one of at least two alternative and detectible stimuli, one of which is deemed to be correct. If the subject responds to the correct stimulus, it receives a reward (Milgram, 2003). In addition to discrimination ability, performance on this task assesses olfactory sensitivity as well as learning and memory capacity. Dogs can learn a number of different odors and discriminate between them. The rate of learning increases as more target odors are added to training (Williams and Johnston, 2002). As an illustration of their discrimation capacity, dogs can detect a target odor that is mixed with large quantities of an extraneous odor. They can maintain memory for a target odor for at least 4 months (Johnston, 1999). Dog-specific traits, such as age, sex, or behavioral traits may influence olfactory discrimination. For example, there is evidence for an age-related effect on olfactory discrimination (Salvin et al., 2012) which may be comparable to the age-related effect on visual discrimination (Milgram, 2003). Our research attempted to determine whether sex, coat color, or emotional resilience influence

olfactory discrimination in Labrador Retrievers, as well as evaluate generalization from trained target odors to chemically related compounds. This information can then be used to improve selection criteria and training methods for candidate IDDs.

Materials and Methods

Test apparatus

The testing apparatus (CanCog Technologies, Toronto, Canada) was a 66 cm x 178 cm x. 91 cm plastic chamber based on a Toronto General Test Apparatus for canines (Figure 16; See Phase III). The chamber was positioned on a cart approximately 64 cm from the ground, equipped with a ramp measuring 114 cm x 58 cm connecting to the rear of the chamber for access. Stainless steel bars separated the portion in which the dog remained (measuring 66 cm x 127 cm x 91 cm) from the area where stimuli were presented (measuring 66 cm x 46 cm x 91 cm). The experimenter sat in front of the testing chamber, visually separated from the dog by a one-way mirror. The front wall of the chamber contained a hinged door measuring 20 cm high. A black, sliding Plexiglas tray with three wells was used to present stimuli by sliding the tray into the chamber through the hinged door. The stainless steel bars could be adjusted for each dog to create three openings allowing the dog to obtain food rewards from the wells. Approximately 2 cm of Pup-Peroni™ Original bacon flavor treats were used as rewards. The chamber and equipment were wiped with a dry cleaning towel in between subjects. After all subjects completed testing for the day, the chamber was cleaned with neutral table cleaner disinfectant solution (1.6 oz Virkon powder dissolved in 20 oz of water).

Data was collected using DogCog™ software (CanCog Technologies, Toronto, Canada) on a computer running Windows 7 interface. The software was used to randomize the location of the rewarded well on each trial, signal the start of a trial with a tone, and time response latencies and inter-trial intervals. A keystroke recorded the dogs' choice on each trial.

Stimuli presentation

The odors were contained inside of 10 cm x 5 cm plastic, visually-identical egg-shaped containers ("eggs") with two pin-hole sized perforations on the top. Depending on the substance, odors could be in a liquid or solid state. Liquid odorants were dispensed by glass syringe onto nylon bags. Solid odorants were placed inside of the nylon bags. An egg containing only an empty nylon bag served as a "blank." In each case, one nylon bag was placed inside one egg. The eggs were positioned horizontally and attached with Velcro to plastic 10 cm diameter coasters, with the holes facing the dog. The two eggs were visually identical and only differed by the odor contained within. Individual eggs were assigned to a particular odorant and were not used for any other odorant.

Test procedures

Initial discrimination: Dogs were tested on a two-choice simple discrimination task to establish whether discrimination between objects on the basis of odor could be established. The initial odor discrimination task assessed each dog's ability to discriminate between vanillin (S+) and an ethanol vehicle (S-). The object discrimination phase consisted of 20-trial sessions, with 30 s inter-trial interval (ITI). The olfactory discrimination (vanillin/ethanol and AN/blank) test criteria was 1 day of 16/20, and then 2 days that add up to at least 28 (out of 40).

Stimulus transfers: After reaching criterion on the initial vanillin/ethanol discrimination, stimulus transfer tests were implemented in which either the S+, S-, or both were replaced with a different odor. In the first stimulus transfer task pure ammonium nitrate (AN) became the rewarded odor and a "blank" became the unrewarded stimulus. The rewarded egg contained 5 g of AN inside of a nylon bag and responses to this egg revealed a food reward inside the well underneath. The "blank" egg was identical and contained an empty nylon bag. No correction procedure was used from this point on. Once criterion was again met with the new stimuli, the first negative stimulus transfer occurred in which the blank egg was replaced with an egg containing 5 g of Iraqi soil inside of a nylon bag. Two days of testing continued with these stimuli, followed by an additional negative stimulus transfer in which the S- was replaced with amyl acetate for two more days of testing. This last pair of stimuli, AN (S+) and amyl acetate (S-), became the baseline stimulus pair for the following phase.

Probe tests: Upon reaching criterion with the baseline stimuli, a series of probe tests using novel odor pairs began. Probe test sessions were implemented in order to assess generalization from training with pure AN to commercial grade fertilizer AN (FAN), as well as to other structurally related chemicals in which equimolar quantities of either the ammonium or nitrate chemical moiety were presented. Probe test sessions consisted of a normal baseline session of paired AN (S+) and amyl acetate (S-) presentations, with probe trials consisting of novel odor pairs randomly inserted throughout the sessions. Probe trials were never rewarded in order to minimize the possibility of rapid within-session learning. *Our hypothesis was that dogs trained on AN would be able to respond above chance to chemicals containing either an ammonium (NH_4) or nitrate (NO_3) salt form*.

Odorant pairs

The following odor pairs were used in the olfactory discrimination phase. Chemical moieties present in AN (ammonia - NH_4 and nitrate - NO_3) are identified as well.

- Vanillin : Ethanol
- Ammonium Nitrate (NH_4NO_3) : Nylon
- Ammonium Nitrate (NH_4NO_3) : Camp Victory soil
- Ammonium Nitrate (NH_4NO_3) : Amyl Acetate (banana)
- Ammonium Nitrate Fertilizer (NH_4NO_3) : Menthol
- Silver Nitrate ($AgNO_3$) : Sodium Sulfate
- Ammonium Chloride (NH_4Cl) : Ferric Chloride
- Ammonium Sulfate ($(NH_4)_2SO_4$) : Ascorbic Acid
- Sodium Nitrate ($NaNO_3$) : Calcium Chloride

Data analysis

For the initial odor discrimination and stimulus transfer tasks, summing the total number of errors committed up to and including the criterion day was used to calculate error scores. A multivariate ANOVA was used to determine whether sex or coat color was significantly associated with olfactory discrimination learning test scores. A multivariate ANOVA was also used to determine whether NCSU ERT score or anxiety phenotype ("worst" or "non-worst") in the open field was significantly associated with olfactory discrimination learning test scores. We also examined whether performance on the object discrimination (Phase III), object reversal (Phase III), DNMP (Phase IV), and olfactory discrimination learning tasks were predictive of one another.

Results

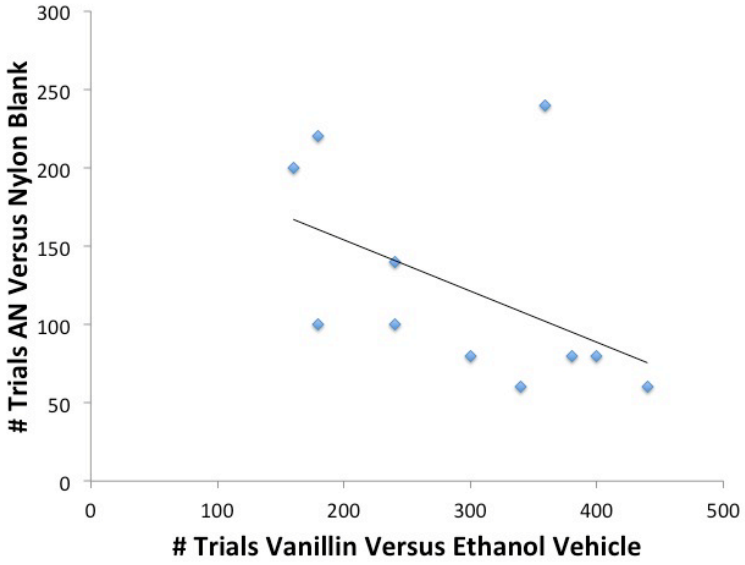
Vanillin olfactory discrimination test

The olfactory discrimination data are presented in Tables 18-20. Except for 'Piper', a dog with poor food/work motivation, all dogs (15/16) completed the vanillin odor discrimination task. The mean (± SEM) number of trials needed to reach criterion was 295.9 ± 24.3. The mean (± SEM) error rate for the vanillin olfactory discrimination test was 32.3 ± 0.8%. Individual animal data (mean ± SEM) are presented in Table 18. There were no significant effects of sex, coat color, or anxiety phenotype on either the number of trials required to reach criterion (Table 20) or the observed error rates (data not shown). Likewise, there was no association between the age of the dog and performance on the vanillin olfactory discrimination test (data not shown). Some, but not all, dogs had received odor-imprinting training at the K2 facility prior to their arrival at NCSU. Odor training did not influence the number of trials needed to acquire the vanillin olfactory discrimination test (Table 20). We also evaluated whether performance on previous cognitive tests correlated with the acquisition rate of the vanillin olfactory discrimination test. We found that performance on the prior tests (object discrimination, reversal task, delayed non-match to position [DNMP]) did not have any predictive value for olfactory discrimination in this cohort of dogs.

AN olfactory discrimination test

The AN olfactory discrimination data are presented in Tables 19-20. As before, one dog ('Piper') was not tested on the AN olfactory discrimination test because of poor motivation. Because of time constraints related to their slower transition through previous cognitive tests, four additional dogs (Dakota', 'Jimmy', 'Reno', and 'Rip') were unable to transition to this phase of the project. The AN olfactory discrimination work was completed in three parts. In part 1, dogs were trained to discriminate between AN and a nylon mesh blank. In part 2 the dogs discriminated between AN and a 5 g soil sample collected from Camp Victory Iraq (see Dorman et al., (2012) for additional details about the soil sample). In part 3, the dogs were trained to discriminate between AN and amyl acetate, a chemical with a marked banana odor. 10 dogs completed all three of these phases. One dog ('Baxter') only completed part 1 of the AN olfactory discrimination test. The overall number of trials needed to reach criteria in part 1 was 123.6 ± 20.0. The mean (± SEM) error rate for the AN olfactory discrimination test was 24.3 ± 2.2%. Individual animal data (number of errors and trials to reach criterion) are presented in Table 19. Similar to our experience with a vanillin-based olfactory discrimination test, there were no significant effects of coat color or anxiety phenotype on either the number of trials required to reach criteria (Table 20) or the observed error rates (data not shown). Likewise, there was no association between the age of the dog and performance on the AN olfactory discrimination test (data not shown). Prior odor training at K2 did not influence the number of trials needed to acquire the AN olfactory discrimination test (Table 20). We did observe a marginally statistically significant effect ($p = 0.0645$, Welch's test) of sex on the number of trials needed to acquire the AN olfactory discrimination test. As can be seen from Table 20, males generally performed poorer on this test than did female dogs. We also evaluated whether performance on previous cognitive tests correlated with the acquisition rate of the AN olfactory discrimination test. As before, we found that performance on the prior tests (object discrimination, reversal task, delayed non-match to position [DNMP]) did not have any predictive value for olfactory abilities in this cohort of dogs. An inverse linear relation between the number of trials needed to reach criteria on the vanillin olfactory discrimination test and AN trial number was seen (Figure 22).

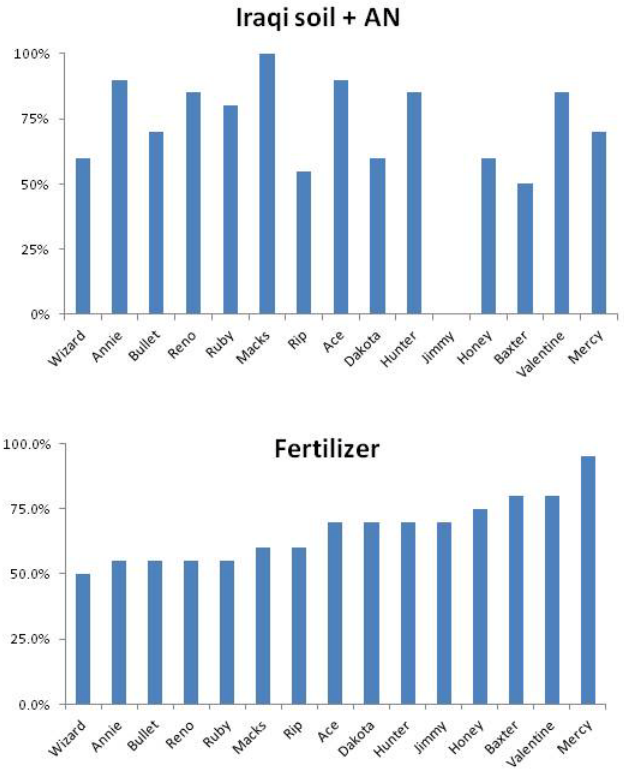
Figure 22. Scattergram showing the statistically significant ($p = 0.0062$, $r^2 = 0.60$) inverse relationship between trials to acquisition of a vanillin- and AN-based test of olfactory discrimination.



AN Generalization

Another goal of this project was to evaluate whether dogs could transition their olfactory discrimination behavior from purified AN to a fertilizer that contains 34% nitrogen as AN. This is equivalent to > 95% pure AN. All dogs had reached criteria on purified AN prior to the conduct of this experimental phase. No effect was seen on sex or coat color so data were pooled in this analysis. The mean (± SEM) correct response rate for the AN/blank (nylon) (last 60 trials only), AN/Camp Victory soil, and AN/amyl acetate odor pairs in the olfactory discrimination test were 81.7 ± 1.7, 80.3 ± 3.1, and 85.8 ± 1.3%, respectively. These data demonstrated the ability of the dogs to discriminate between purified AN versus other odor sources. In order to reduce the impact of learning, the dog's ability to detect fertilizer was based on responses to a 20 trial probe, run in extinction, where the dog was presented with a AN-based fertilizer and menthol odor pair (this is used to minimize dogs responding to an odor – 'no odor' pair). The mean (± SEM) correct response rate for the fertilizer-grade AN/menthol odor pair was 67.2 ± 4.9%. We also analyzed the results of the AN/Camp Victory soil, and AN/amyl acetate, and fertilizer-grade AN/menthol odor pairs for the first 20 trials (i.e., similar to the AN-based fertilizer trial). The mean (± SEM) correct response rate for the AN/Camp Victory soil, AN/amyl acetate, and fertilizer-grade AN/menthol odor pairs were 73.8 ± 4.8, 87.2 ± 1.7, and 67.2 ± 3.3%, respectively. Performance on the fertilizer-grade AN/menthol odor pair was significantly poorer than that seen with the other two odor pair combinations (ANOVA $p = 0.0019$, post hoc Tukey's $p < 0.05$) but remained significantly higher than chance. Figure 23 shows the performance of individual dogs on these olfactory discrimination test that used AN/CV and fertilizer-grade AN/menthol odor pairs.

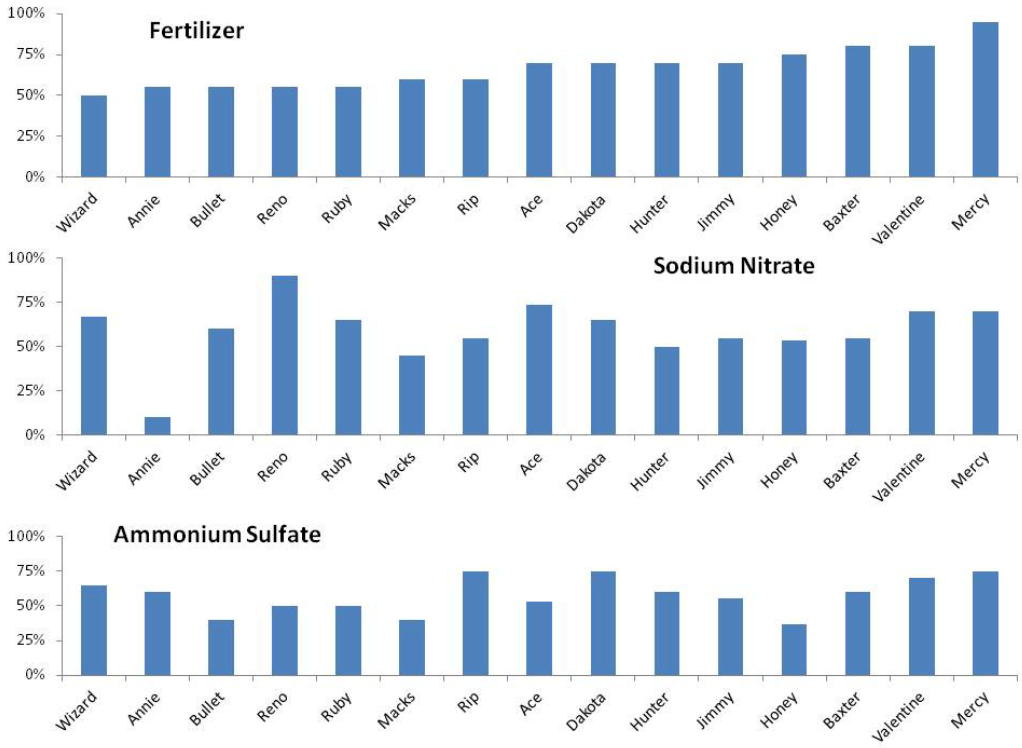
Figure 23. Dogs used in this experiment were previously trained to discriminate purified AN versus a second odorant. Performance on an olfactory discrimination test evaluating the dog's ability to discriminate between AN (5 g) with 100 g Iraqi soil (S +) and an unrewarded odor (100 g Iraqi soil alone, S -) is shown (top). Performance on an olfactory discrimination test evaluating the dog's ability to discriminate between fertilizer grade AN (S +) following training with purified AN and an unrewarded odor (menthol, S -) are also shown (bottom). Mean response rates are presented (minimum 20 trials for each odor pair).



Chemical Probe Trials

We also wanted to evaluate the ability of dogs previously trained on AN to detect odorants chemically related to AN. For these experiments we identified simple organic chemicals that contained either an ammonium or nitrate functional group. Figure 24 shows the results of these experiments. We have included the results from the fertilizer grade AN experiment for comparison. The mean (± SEM) correct response rate (for the final 20 trials) for the ammonium sulfate /ascorbic acid and sodium nitrate/calcium chloride odor pairs were 57.7 ± 3.3 and 57.2 ± 5.1%, respectively.

Figure 24. Individual correct response rate (%) for dogs trained with purified AN. A response rate of 50% would be expected if the dog chooses the chemical at a random response rate. Performance on all odors was significantly worse than that seen with purified AN ($p < 0.05$). With the exception of fertilizer AN, all odor pairs were statistically equivalent to chance.



Discussion

The system designed by CanCog™ has been used by other investigators to evaluate cognitive function in dogs using visual discrimination procedures (Milgram, 2003). We report one of the first successful acquisition of an olfactory discrimination task by dogs using the CanCog™ system. Our first set of experiments used vanillin, a chemical frequently used by researchers studying olfaction in people, dogs, and other species. Studies in animals and people show that vanillin preferentially activates the olfactory cortex (Frasnelli et al., 2011, Savic et al., 2002). Different odorants can stimulate other brain regions. For example, acetone, a chemical with odor and irritant properties predominantly activates trigeminal projections from the nasal mucosa (Savic et al., 2002).

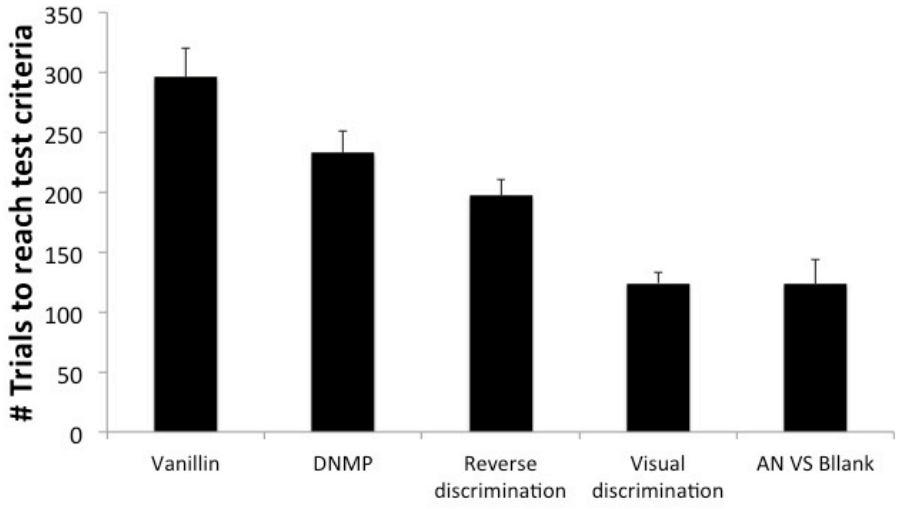
In addition to vanillin's "relatively pure" olfactory stimulus (Frasnelli et al., 2011), we also chose vanillin because we anticipated that it would represent a novel odor for the dogs used in our experiments. One goal was to determine whether other cognitive measures (e.g., visual discrimination), physical characteristics (e.g., sex, coat color, age), or personality traits (e.g., emotional reactivity, anxiety phenotype) could influence or predict novel odor learning in dogs. Of the factors under investigation, previous studies have shown that age and sex may influence olfactory processing and these responses can be chemical specific. For example, men and women's perceived hedonicity (pleasure) of vanillin and certain other odors differ (Seubert et al., 2009). Some studies have shown that in women, odor discrimination scores decline significantly

with age (> 45 years of age), whereas no effect of age is seen in similarly aged men (Boesveldt et al., 2008; Stuck et al., 2006).

In our studies, we found that physical, cognitive, and emotional traits did not affect the ability of a dog to learn a vanillin olfactory discrimination task. The lack of a sex- or age-dependent effect is not unexpected given the fact that our dogs were a relatively homogenous population of young dogs. We also did not see an effect of prior odor imprinting work performed at K2 on the ability of dogs to acquire the vanillin or AN olfactory discrimination test.

Among the cognitive tests used, acquisition of the vanillin olfactory discrimination test proved to be one of the most difficult (Figure 25). Although it would be expected that dogs would show a superior performance on an olfactory task than visual based on their exceptional olfactory system, it is likely that prior training on a visual discrimination task may have hindered performance on the subsequent olfactory task. Dogs began the olfactory discrimination task immediately upon completing the visual discrimination phase, requiring an inhibition of responding based on visual properties and a shift to attending to olfactory cues. Other investigations have shown that the order of the cognitive tasks given to a dog can influence learning effectiveness. An adequate comparison of olfactory versus visual discrimination learning would require two cohorts of dogs in which the order or modality training is counterbalanced. Interestingly, the ability of dogs to learn the AN olfactory discrimination test was inversely related to their ability to acquire the vanillin olfactory discrimination test. Once dogs were familiar with the odor discrimination test they rapidly learned new odorant pairs. This observation is consistent with findings that performance is facilitated by increasing the number of odors to learn in dogs (Johnston, 19999) and rats (Peña et al., 2006).

Figure 25. Total trials associated with dogs reaching the test criteria. Some tests (e.g., DNMP) were terminated after a predetermined number of trials



Our studies also focused heavily on AN because many of the improvised explosive devices encountered in the Middle East are based on this oxidant source. Since some, but not all dogs, had undergone a variable duration of prior odor imprinting we wanted to begin these studies with a pure form of the chemical. Once dogs learned to discriminate the odor of AN from the vinyl-based blank netting material used to hold the solid AN we then explored the ability of dogs to discriminate AN from Middle Eastern soil (collected at Camp Victory, Iraq) and amyl acetate.

These experiments investigated how a stimulus–response–reinforcer relationship in one context (AN/vinyl blank) migrated to other odor pairs (context shift effect). Context shifts are often known to reduce animal olfactory performance (Thomas et al., 1993). We found that dogs were able to maintain the ability to discriminate pure AN from other unrelated odor sources. This ability was unaffected by the dog's physical, cognitive, and emotional traits or prior odor imprint performed at K2.

The next step in our experiment investigated whether dogs trained to discriminate pure forms of AN could generalize this behavior to fertilizer grade AN (which contains enough AN to provide 34% nitrogen in the fertilizer). A chemical analysis of the fertilizer-grade AN was performed by James N. Thomasson III, of the Energetics Test and Evaluation Division, Naval Surface Warfare Center. The results of this analyses indicate that the sample was water soluble and consistent with a prilled material having a composition of >95% AN (by weight). Analysis of the sample by x-ray fluorescence did not suggest significant metal content but did suggest the presence of low levels of either calcium or silicon suggesting minimal quantities of either limestone (mostly mixture of various crystalline forms of calcium carbonate) or silica (silicon dioxide). The prilled material had high surface sheen, likely coated with an organic material (typically very low concentrations). We found that overall, dogs trained on pure AN could detect fertilizer grade AN at a success rate slightly greater than chance. It should be noted that some dogs maintained a higher degree of success with this task suggesting that olfactory capabilities vary significantly within a breed.

Finally, we wanted to explore the physico-chemical basis for the detection of AN. Most target odors are composed of many different vapor compounds and dogs can probably detect many compounds in such mixtures (e.g., Johnston, 1997; Williams, et al., 1999). In its relatively pure chemical form, the AN molecule is extremely simple, being composed of equimolar quantities of the ammonium cation (NH_4+) and nitrate anion ($NO3^-$). One question that we sought to answer was whether the dog's detection of AN is based upon recognition of either the ammonium or nitrate moiety. To this end, we used a series of purified chemicals that represent different ammonium- or nitrate-based salts in dogs that could discriminate AN. In our studies, the ability of dogs to detect these other chemical salts was consistently equivalent to chance (i.e., 50% correct response rate), suggesting that the odor signature for AN differs from the other ammonium or nitrate containing salts. This finding, as well as our results examining the ability of dogs trained on AN to generalize to fertilizer grade AN, has important implications for the training of dogs used for IED detection. Our results strongly suggest that ability of dogs to generalize between chemically related simple inorganic compounds is limited even between forms of the same chemical that differ by concentration and extraneous fillers.

One other important observation that arose from these studies is the inability of commonly used screening tests (e.g., the USMC ERT, see Phase I) to predict olfactory function in Labrador Retrievers under laboratory conditions. Likewise, performance on other cognitive tests also proved ineffective at predicting olfactory function. These results are not entirely unexpected, because brain function and response are highly compartmentalized and rely upon different neural substrates. Once animals were conditioned to the standardized laboratory conditions and personnel, then the environment was very predictable, conditions that would mitigate any effects of fear. Our protocol allowed us to evaluate each dog's olfactory capacity, independent of emotional responses. As in our other experiments, we identified subsets of dogs that could be considered 'fast' or 'slow' learners of an odor-based test. Since IDD performance relies heavily upon the ability of dogs to hunt and detect odors there may be value in developing short-term assessments that can be used to predict olfactory ability in dogs.

PHASE VI. COGNITIVE BIAS

Background

Information processing by humans can be biased by their emotions. For example, anxious and depressed people tend to make negative judgments about events and to interpret ambiguous stimuli unfavorably (MacLeod and Byrne 2006). Similar 'pessimistic' response bias can also be seen in rats that are housed in unpredictable or stressful conditions (Burman et al., 2009; Harding et al., 2004). These and other studies provide evidence that animals experiencing different emotional states following exposure to long-term environmental manipulations show contrasting biases in their judgment of ambiguous stimuli.

Historically, tests of cognitive bias in non-human animals have been aimed at correlating positive and negative affective states of animals to the effect it has on their judgment of ambiguous stimuli (Burman *et al*., 2011). The ultimate goal of the majority of this research has been aimed at both evaluating and improving the welfare of animals (Mendl *et al*., 2009; Burman *et al*., 2011). Some research has related a 'pessimistic' cognitive bias to separation-related behavior in shelter animals (Mendl *et al*., 2010). However, further research conducted on cognitive bias in sheep recognizes a limitation of these tests by demonstrating a learned component that affects results due to repeated ambiguous trials (Doyle *et. al*, 2010). Cognitive bias testing involves a component of spatial learning. A task known as a delayed non-matching to position task (DNMP) has specifically been used to evaluate learning based on spatial cues (see Phase IV). In this task, the subject is presented with a single object on one side of the testing field, which yields a reward when the subject moves the object. After a delay of time, the subject is presented with two identical objects on both sides of the testing field. This time, the subject receives the reward when it moves the object on the side opposite that of initial object presentation (Milgram *et al*., 1999).

The goal of this research is to evaluate the 'personality' of the Labrador Retriever to determine whether certain behavioral traits may be predictive of future function as a working dog. We also want to evaluate whether performance on a spatial cognitive bias test is correlated to the results of our previous DNMP test gathered on the same subjects over a 3-month period. If the correlation proves to exist, the test of cognitive bias could prove to be a more concise, but equally informative, alternative method for IDD candidate selection as it relates to spatial learning.

Materials and Methods

Cognitive Bias Test

On the same day as the cognitive bias testing (Cohort 1 only), dogs also participated in either DNMP testing or odor discrimination in the CanCog apparatus and received fifteen minutes of physical activity outdoors, off-lead, in a fenced area. Two dogs were fasted for 19 hours prior to conducting the cognitive bias test due to a lack of food reward drive. Further details about animal husbandry and the DNMP are available elsewhere in this report.

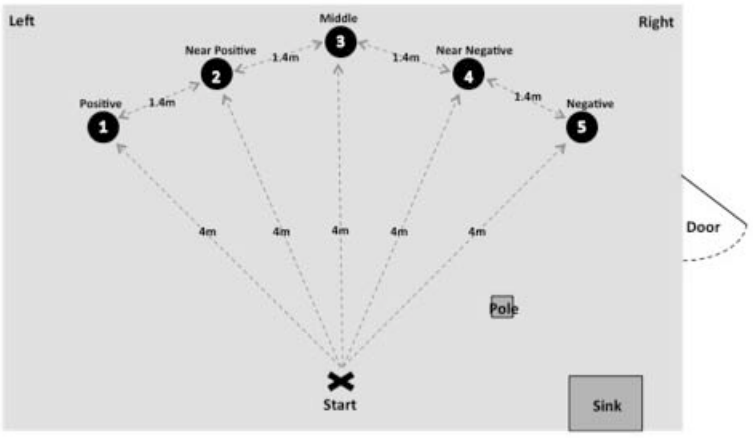
The cognitive bias test was conducted using previously published methods (Mendl et al., 2010). The test room was a vacant CVM LAR animal room that measured 4.7 m x 7.2 m (see Figure 26). Locations where bowls were to be placed were pre-marked with red electrical tape and labeled 1 through 5, from left to right, for ease in identifying locations between trials. The start position was also pre-marked and was labeled as "Start". Two researchers were present with the dog throughout the test. Before the start of each trial, the dog was put on a lead and held by one of the

researchers outside of the room with the door closed while the other researcher placed a large stainless steel food bowl with or without a small piece of a food reward (Pup-peroni) reward (depending on trial type) inside it at one of five pre-determined locations 4 m in front of the designated starting position. The dog was then led into the room to the start position, the leash was unhooked from the collar, and the dog held by the collar. The researcher would then simultaneously release the collar and give the command "go" to allow the dog to approach the bowl. If the dog did not leave the start position on the initial command "go", a few seconds were allowed to elapse, then the command was repeated. If the dog still did not leave the start position, the command "hunt it up" was given. If the dog still remained at the start position, no further commands were given. The latency to reach the bowl, defined as the time elapsed between release from the researcher and the dog putting its head into the bowl or looking into the bowl, was recorded for each trial using a stopwatch.

Dogs were first trained that when the bowl was placed at one ('positive') location on one side of the test area, it would contain food, and when it was placed at another ('negative') location on the opposite side of the test area, it would be empty. For half the dogs, the positive location was on the right hand side as they faced the test area, and for the other half it was on the left. Initially, each dog received two consecutive positive trials (bowl placed in the positive location with a food reward in it) followed by two negative trials (bowl placed in the negative location with no food reward). Subsequently, positive and negative trials were presented in a pseudorandom order, with no more than two trials of the same type being presented consecutively. During the first 10 training trials, if the dog did not approach the bowl within 30 seconds, the test trial was terminated and the dog was taken to the bowl (regardless of whether a reward was present). All dogs received a minimum of 15 training trials (any trial that terminated at 30 seconds was still considered a valid training trial). In order to proceed to the testing phase, dogs had to have had at least 15 training trials and met the criteria for a learned association between the positive location and a food reward. To meet this criterion, dogs had to have a shorter latency to reach the positive location such that their longest latency to the bowl on 3 consecutive positive trials was shorter than the latency to reach the bowl on 3 consecutive negative trials. On each trial, dogs were given a maximum of 30 seconds to visit the bowl. If they had not visited it by this time, the trial was terminated, a time of 30 seconds was recorded, and the next trial was initiated.

Testing began immediately after the learning criterion was achieved. Test (probe) trials were identical to training trials except that the bowl (without a food reward) was placed at one of three ambiguous locations equally spaced 1.4 m apart along an arc 4 m from the dog's start position, and between the positive and negative locations (Figure 26). The three locations were: near-positive (NP: 1.4m from the positive and the middle locations), middle (halfway along the arc, 2.8 m from the positive and negative locations), and near-negative (NN: 1.4 m from the negative and middle locations). Three probe test trials were presented at each location (nine test trials in total) in the following order: M, NP, NN, NP, NN, M, NN, M, NP (each location was presented first, second or third in each block of three test trials). The purpose of the test trials was to investigate how dogs responded to these ambiguous locations and whether they tended to run quickly to them (indicating anticipation of a food reward – an 'optimistically' biased judgment of the ambiguous cue) or more slowly (indicating lower anticipation of food – a 'pessimistically biased judgment).

Figure 26. Cognitive bias testing facility layout for a dog trained for the left side bowl to be positive (P) and the right side bowl to be negative (N).



The testing phase began with two consecutive positive trials followed by two consecutive negative trials. Following that, each probe test trial was separated from the next by four pseudorandom training trials (positive and negative locations), identical to those used in the training phase, in order to maintain and reinforce the associations between the positive and negative locations and reward. After the last ambiguous location test trial, four more training trials were run and a final trial was then conducted in which an empty bowl was placed in the positive location. The purpose of this was to determine if dogs ran just as fast to an empty bowl in this location as to the usual baited bowl and hence were not relying on odor cues to detect whether the bowl was baited. The entire test phase involved an additional 40 training trials, 9 probe test trials, and one empty bowl trial for a total of 50 additional trials beyond the initial training phase.

Data analysis

Mean latencies to get to the bowl during each of the three types of test trial (NP, M, NN), and during training trials (P, N) were calculated for each dog. To control for differences in dog size and running speed, each dog's test trial latency was adjusted according to its mean 'baseline' latencies during training trials (Mendl et al., 2010). The adjusted score is calculated by:

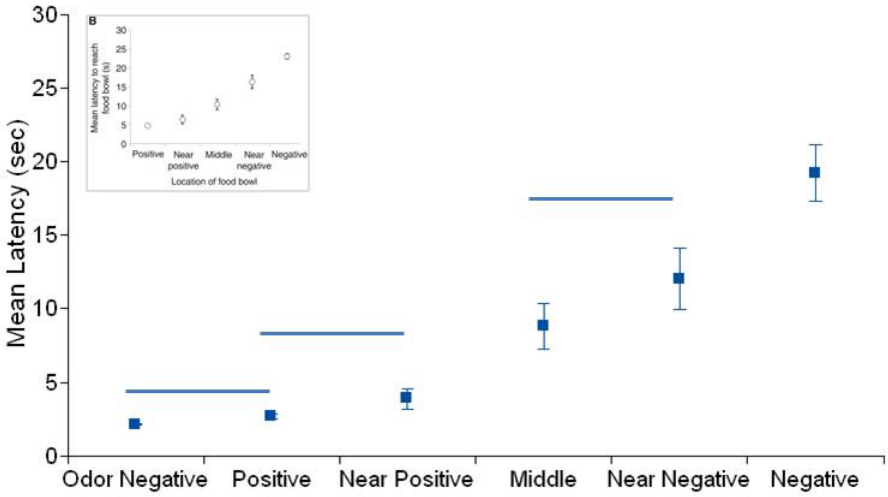
where, is the mean latency for a given position.

This adjusted score expresses all probe test latencies as a percentage of the difference between each dog's baseline mean latencies to the positive and negative locations. Overall mean values for a parameter were calculated before completing data analysis. Latency data were initially analyzed using ANOVA however, the data did not have equal variances. Log transformation of the data was inadequate; therefore, this data was analyzed using non-parametric statistics (Wilcoxon method). A repeated measure ANOVA was used to assess whether coat color or sex was associated with the change of adjusted latencies across consecutive trials.

Results

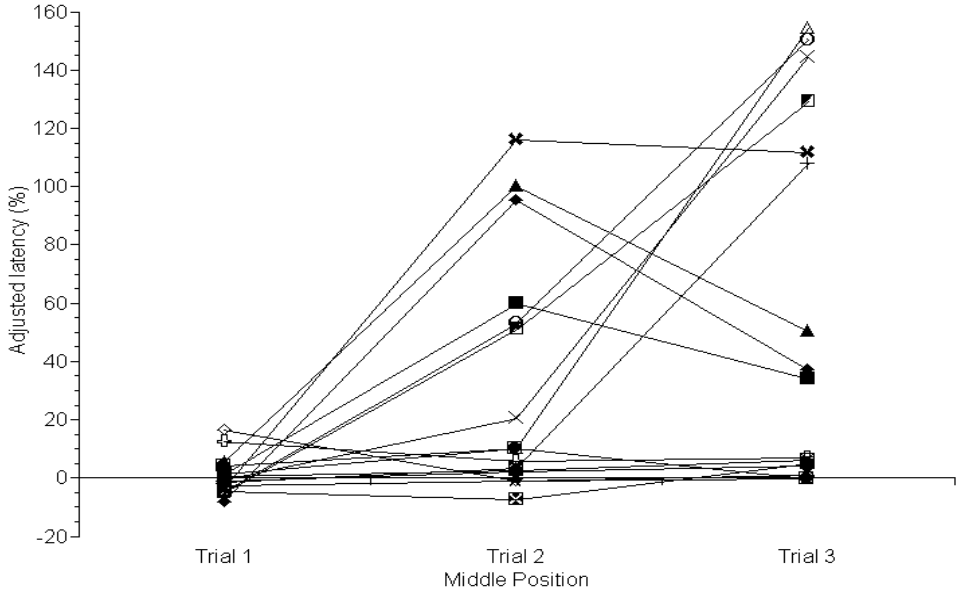
The number of training trials to the cognitive bias test for all dogs varied from 15 to 43 training trials (Table 21). The mean number (± SEM) of training trials was 21.9 ± 2.1. The dogs segregated into two groups depending on their total number of individual training trials. They were dogs that took ≤ 19 training trials to meet criteria (fast learners) and dogs that took ≥ 19 training trials to meet criteria (slow learners, Table 21). Throughout this phase, the bowl was placed at both the positive and negative positions a total of 20 times each per dog, at the near positive, middle, and near negative position a total of 3 times each per dog, the odor negative position (positive position with no reward) 1 time per dog. Mean latencies (± SEM) were calculated for each bowl position during the cognitive bias testing phase and can be seen in Figure 27. The latency to the odor control (bowl placed in the positive position, but with no reward) was statistically identical to the time to reach the positive location demonstrating that the dogs were not using odor cues to determine whether or not to approach the bowl.

Figure 27. Unadjusted mean (± SEM) latencies observed during the test phase on trials where the bowl was placed at the positive and negative training locations, and at the three test locations: near positive (NP), middle (M), near negative (NN). Inset shows data for 24 dogs (age range: 9–108 months) housed at two UK animal shelters (from Mendl et al., 2010). Bars shown above the data indicate data pairs that were statistically equivalent (Wilcoxon test). All other data pairs would be statistically different from each other.



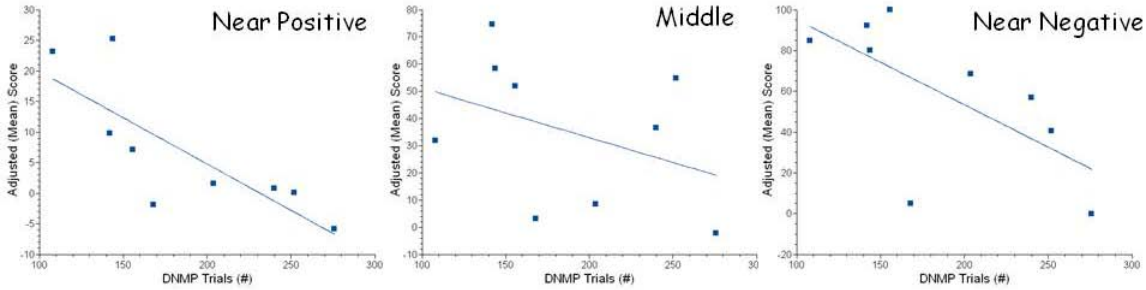
Middle latency curves were developed based on the adjusted raw data of individual middle trials for each dog and can be seen in Figure 28. During the first middle trial, the entire cohort's adjusted latencies are grouped and indicate a generally fast speed to this location.

Figure 28. Middle adjusted latency curves (mean ± SEM).



No correlation was observed between the adjusted mean latencies for near positive, middle, and near negative positions and either the number of trials or percent errors in the object discrimination or reversal tasks (data not shown, see Phase III). Likewise, there was no significant correlation between the percent errors in anxiety phenotype ('Worst' or 'Non-Worst') or NCSU ERT score and any of the ambiguous locations. A significant linear correlation was found between the adjusted mean latencies of the near positive ($p = 0.0009$, $r^2 = 0.56$) and near negative ($p = 0.013$, $r^2 = 0.39$) ambiguous positions and the number of DNMP trials ($p \leq 0.05$; all dogs). This significant linear correlation remained present when the data from only the subset of dogs (n = 9) that completed the DNMP task were analyzed (Figure 29). In this case, a statistically significant correlation was seen for the near positive ($p = 0.0099$, $r^2 = 0.64$) and near negative ($p = 0.0452$, $r^2 = 0.43$) ambiguous positions and the number of DNMP trials ($p \leq 0.05$; DNMP subset). A near significant correlation was found between adjusted mean latency of the middle ambiguous position and the number of DNMP trials ($p = 0.097$, $r^2 = 0.20$).

Figure 29. Significant linear correlation ($p < 0.05$) seen between the adjusted mean latencies of the near positive and near negative ambiguous positions and number of DNMP trials needed to reach criterion on this task. Data also shown for the middle position (not significant).



Discussion

Cognitive bias testing in dogs is highly correlated with performance on a spatial learning task (DNMP) that is a reflection of cognitive function. As superior cognitive function may be indicative of a dog's ability to learn and retain knowledge of tasks over time, and function during stress, this is a quality desired in selecting dogs for IED detection work. The cognitive bias test used here is easy to perform, relatively quick, and requires very little pre-training or adaptation to the test arena. As such, it is a complementary and expedient addition to the ERT for screening dogs.

In addition to being reliable and expedient, screening tests should also be generalizable to other test environments. Mendl et al., (2010) previously reported that the number of training trials required by 24 dogs of varying breed, size, and sex to reach criteria on the cognitive bias test was 21 to 61 (mean ± SEM; 29.42 ± 8.86). For our cohort of Labrador Retrievers, the number of training trials ranged from 15 to 43 (mean ± SEM; 21.94 ± 8.24). Further investigation into breed differences as it relates to training time may be warranted in order to determine if certain breeds consistently produce faster training results. However, the criteria of the current cognitive bias training protocol (Mendl et al., 2010), in which dogs "were deemed to have learned an association between bowl location and food reward when, for the preceding three positive trials and the preceding three negative trials, the longest latency to reach the positive location was shorter than any of the latencies to reach the negative location" appears be too weak. Some dogs only minimally met the test criteria. Only later in testing could a distinct difference be seen in the responses to positive and negative positions. Thus, we feel that the criteria of the current cognitive bias test protocol should be revaluated for future tests in order to ensure all dogs have sufficiently learned the task before proceeding to the testing phase.

Similar to other reports, we found spatial differences with position. As seen in Figure 27 above, with increasing distance from the positive bowl position, there was an increase in mean latency to the bowl, making our data qualitatively similar to that reported by Mendl et. al, 2010. The odor control data (bowl placed in the positive position, but with no reward) demonstrates that the cohort of dogs was not using odor cues to determine whether or not to approach the bowl.

Others have ascribed their similar results to personality differences, identifying animals as 'pessimists' or 'optimists'. We found that results were not associated with other traits that may indicate personality or temperament including performance on the USMC ERT or anxiety phenotype from the OFT. We did find that performance on the cognitive bias test was highly correlated with spatial learning ability (as assessed by the DNMP test). As seen in Figure 28, using the results of only the middle trial as it represents a neutral location between the positive and negative positions, the cohort of Labrador Retrievers behaved in a few different ways. During the first middle trial, the entire cohort's adjusted latencies are grouped and indicate a generally fast speed to the location. During the second middle trial, about 10 of the dogs maintain a generally fast speed to the middle position, while 6 of the dogs dramatically slow their speed to a moderate pace. On the third, and final, middle trial, 7 of the dogs continue to maintain a relatively fast pace, while 3 dogs investigate the position at moderate speed, and the remaining 6 dogs slow down even further. We perceive these results to indicate that dogs, over time, learn that this bowl position is unrewarding. This interpretation is in agreement with data collected in sheep which "shows a significant decline in the total number of approaches to ambiguous positions over time because the sheep learned that these ambiguous locations were unrewarded" (Doyle et. al, 2010).

Further evidence that spatial learning influences performance on the cognitive bias test comes from our analysis showing a correlation between the number of DNMP trials to meet criteria and the adjusted latency scores of the ambiguous locations. Spatial learning is believed to occur by two means, by egocentric cues or allocentric cues. Utilizing egocentric cues for learning occurs by reference to the subject's body position; whereas use of allocentric cues occurs by reference to the position of an external referent or landmark (Milgram et al., 1999). The DNMP task relies on egocentric cues for learning and, based on the significant correlation between those data and the cognitive bias data, we believe that spatial learning is a key factor in a dog's decision to approach the bowl when in an ambiguous position.

Based on our results, while we cannot rule-out a 'pessimistic' or 'optimistic' cognitive effect on our results, we feel the spatial learning component should not be overlooked. From this knowledge, we feel that the short-term cognitive bias test could be a suitable alternative to the longer DNMP task to evaluate dogs on their cognitive aptitude for spatial learning. This test could then be used in the future to enhance other criteria for selection of working dogs

PHASE VII. APPLICATION OF REMOTE TELEMETRY TO A NOVEL OPEN FIELD TEST OF OLFACTION

Background

Several behavioral attributes can contribute to the success of an improvised explosive device detector dog (IDD). These include the trainability, motivation for sniffing, acuity of the sense of smell, ability to focus on searching and ignore distracting stimuli, temperament, eagerness to search without being discouraged by a lack of success, and ability to work effectively in a stressful or novel situation (Rooney et al., 2004). Our research has addressed many of these aspects. However, an objective method for measuring the acuity of a dog's olfactory capacity remains elusive.

This research project extends our previous work through the development of a novel open field test. There are important differences that distinguish this open field test from the one used in our earlier studies (Phase II). The present olfaction assessment tool has a considerably larger search area (6 X larger in size), animals wore telemetry vests, the test duration was twice as long (20 min), and two odorant sources were incorporated into the test arena. Several design considerations were shared by both tests. The dog were allowed to explore the test arenas without direct human involvement, behavior was monitored by videography, and physiological responses (e.g., change in heart rate) were assessed. One important difference is was that the use of remote telemetry in the present test allowed us to also monitor changes in respiration , heart rate, and temperature as they occurred in real time. For purposes of discussion we refer to our canine olfaction assessment test by the acronym COAT.

Adrian (1950) was one of the first to recognize the relationship between the velocity of the air through the nose and olfactory stimulation in the mammalian olfactory bulb. Since this seminal observation multiple studies have clarified this essential relationship between respiration and olfaction using electroencephalogram (EEG)-derived event-related potentials (ERP), brain imaging, single unit recording of mitral cell layer neuron activity, and other methods (Griff et al., 2008; Haehner et al, 2011; Mainland and Sobel, 2006; Sabri et al., 2005). Other studies suggest that changes in the respiratory cycle prepare the olfactory bulb for sensory activity. Freeman and Schneider (1982) noted that the electrical activity in the rabbit olfactory bulb changes prior to inhalation. This change appears to "prime" the olfactory bulb for stimulation. Further, numerous studies have suggested that olfactory information is encoded in the precise timing of mitral cell spiking relative to the respiratory cycle (Phillips et al., 2012). Ultimately, these studies have clearly demonstrated that olfactory perception depends on respiration.

Odor detection in mammals often begins with a 'sniff' (defined as a short, audible breath through the nose, as in smelling something or use the sense of smell, as in savoring or investigating). Indeed, the important role of sniffing in the formation of the olfactory percept has been increasingly recognized among neurobiologists (Buonviso et al. 2006; Kepecs et al. 2006; Mainland and Sobel 2006; Schoenfeld and Cleland 2006). Considering the influence of sniffing on the resultant olfactory percept and on patterns of neural activity throughout the olfactory system, accurate measurement of sniff parameters is paramount. The most important sniff measures are related to the temporal dynamics of the sniff and the resultant airflow volumetrics that deliver the odorant to the olfactory epithelium (Johnson et al., 2006). A typical human sniff has a duration of 1.6 s, an average inhalation velocity of 27 l/min, and a volume of 500 cm^3 (Laing, 1983).

Most studies performed to date have relied on human volunteers and experimental rodent studies. To our knowledge, collection of near-natural olfactory behavior by dogs during their performance of an olfactory task has not been reported. The goal of the present experiment was three fold: (a) assess whether we could collect information about sniffing during odor detection; (b) evaluate the impact of the telemetry jacket on normal dog activity patterns; and (c) evaluate whether the COAT could serve as a screening tool for behaviors critical to IDD function (motivation for sniffing, ability to focus on searching and ignore distracting stimuli, eagerness to search, and ability to work effectively in a novel situation).

Materials and Methods

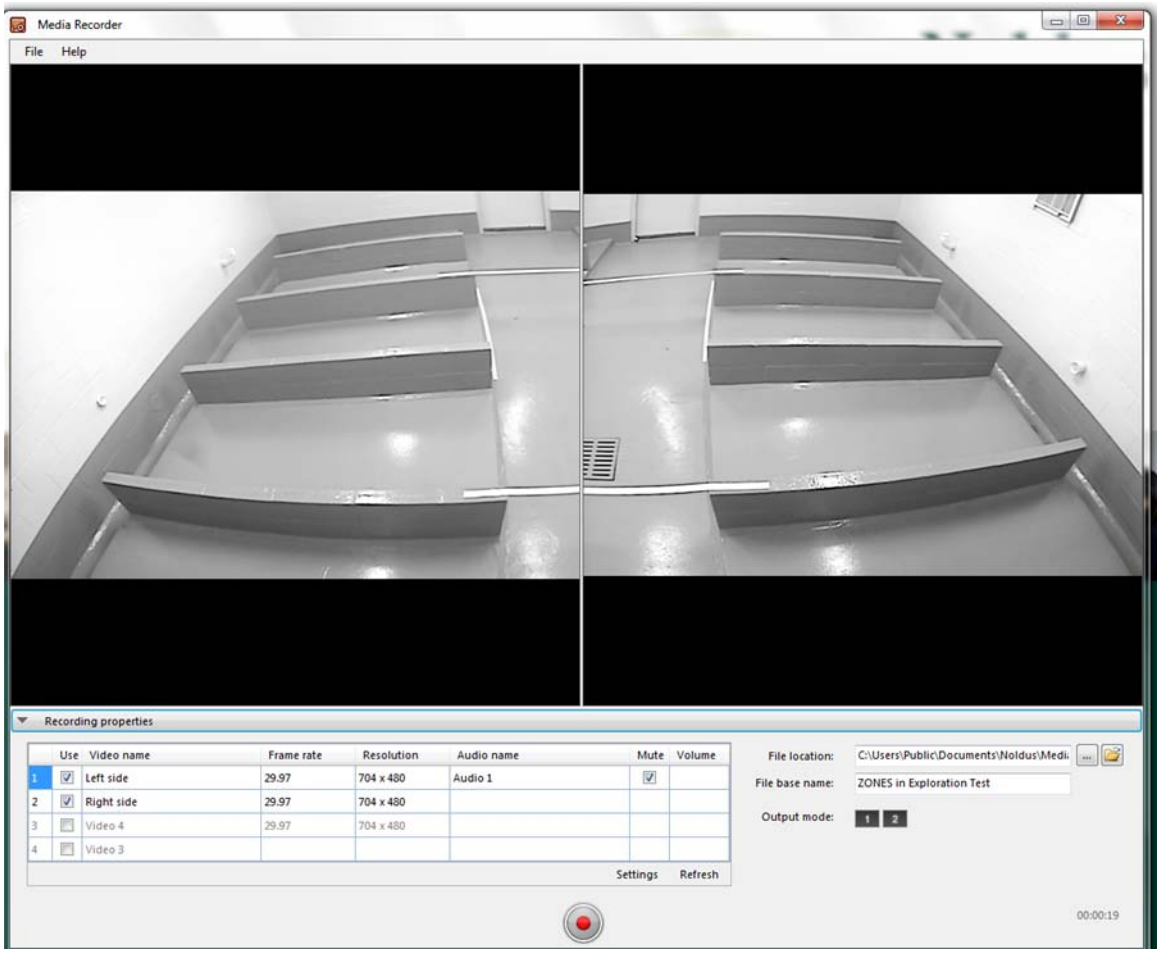
Animals

The current study used 16 Labrador Retrievers aged 1.5-3.5 years (cohort 1). Dogs had varied background training for detection of explosives, but did not meet certification standards for use as IDDs. The dogs had been previously tested in a different open field test (Phase II) and had been trained to respond to certain odors for a food reward in the CanCog apparatus (Phase V).

Open field design

A 6.4 x 7.3 m novel open field was developed with 4 static odor ports spaced evenly within the room (Figure 30). The open field had 3 cinder block walls and a front wall covered with tarps. There were 8 short cinder block partitions remaining from its previous use as a kennel. They were 0.46 m tall and extended from 0.3 m away from the side walls for 2.4 m toward the central aisle. Four static odor ports were constructed of 10.2 cm PVC pipe with PVC gratings covering the port and mounted to the wall at a height of 76 cm. Two of the ports were designed to hold a liquid and contained a removable cup. A remote leash release point was placed in the middle of the front wall. Two speakers mounted on this wall played white noise at ~70 dBA. A total of 4 cameras captured the activity of the dogs as they were exploring the open field. Two Ikegami ICD-49, monochrome IR cameras provided live monitoring of the majority of the room as well as direct storage by Noldus Media Recorder software (Noldus Information Technologies, Leesburg, VA). Two additional Canon ZR960 cameras were mounted on opposite ends of the front wall to record areas (release zone) that could not be viewed by the primary cameras.

Figure 30. Open field as seen with two video cameras acquiring data using Noldus Media Recorder. Four white odor ports are mounted on the side walls. White tape has been temporarily placed to identify boundaries of zones for data analysis purposes. The four quadrants near odor ports were designated A-D, the central aisle was the neutral zone, and the area near the lower edge of the view is the release zone.



Odorants

Liquid from canned tuna fish in vegetable oil (~20 mL) and soiled cat litter (~45 g) were used as two novel odors to be placed in the odor ports. These odors were selected because of their presumed novelty and intrinsic interest. The position of the odorants was alternated based on the testing date. The remaining two odor ports were empty and served as blanks. Before each testing day began, the odors were placed in cleaned ports and allowed to off-gas for a minimum of 30 minutes before exploratory tests were started.

Remote physiology data collection

Physiological activity was monitored using the emkaPACK non-invasive telemetry system paired with IOX 2.8.0.11 collection software and ecgAUTO v 3.10.12 analysis software (emka Technologies, Falls Church, VA). The dogs' hair was clipped along both sides of the chest approximately one week before testing. Dogs were prepared for telemetry recording in an anteroom so that the quality of recording could be verified prior to the test session. On the day of testing, the skin was cleaned with an alcohol wipe and allowed to dry. 1-lead ECG recordings were made by attaching 3M RedDot Ag/AgCl repositionable monitoring electrodes to the chest. The positive lead was placed at the costochondral junction of the sixth rib on the right side, and the negative lead was placed in the same location on the left side of the chest. A neutral lead was placed over the last rib on the right side. A Mesurex skin temperature probe was placed in the axillary region of the left side. The electrodes were held in place with adhesive elastic bandage wrapped around the dog. A Lomir undershirt and vest (Lomir Biomedical, Quebec, Canada) was worn over the electrodes and held an emkaPack transmitter. An embla XactTrace elastic belt fit in the undershirt and fastened around the lower chest to record respiration by electrical impedance changes. The emkaPack transmitter contains an accelerometer activity monitor. Dogs were acclimated to wearing the undershirt and vest for several days while having outdoor exercise. None of the dogs appeared to be disturbed by wearing the telemetry vest and electrodes. Continuous recordings of the electrocardiogram (ECG), respiration, skin temperature, and activity were made while the dog was in the open field arena. These recordings were annotated during collection based on observation of the video monitors.

Odor exploratory test

The open field room was cleaned with Virkon disinfectant diluted with water before each test to reduce olfactory cues from the previous test subject. Each dog was walked on a light, 1.2 m x 1.4 cm nylon leash into the open field room and tethered to the leash release point. The leash was released 30 seconds after the handler had exited the room. This was designed to avoid bias due to interaction with the handler. Video and telemetry recording were carried out for a total of 20 minutes.

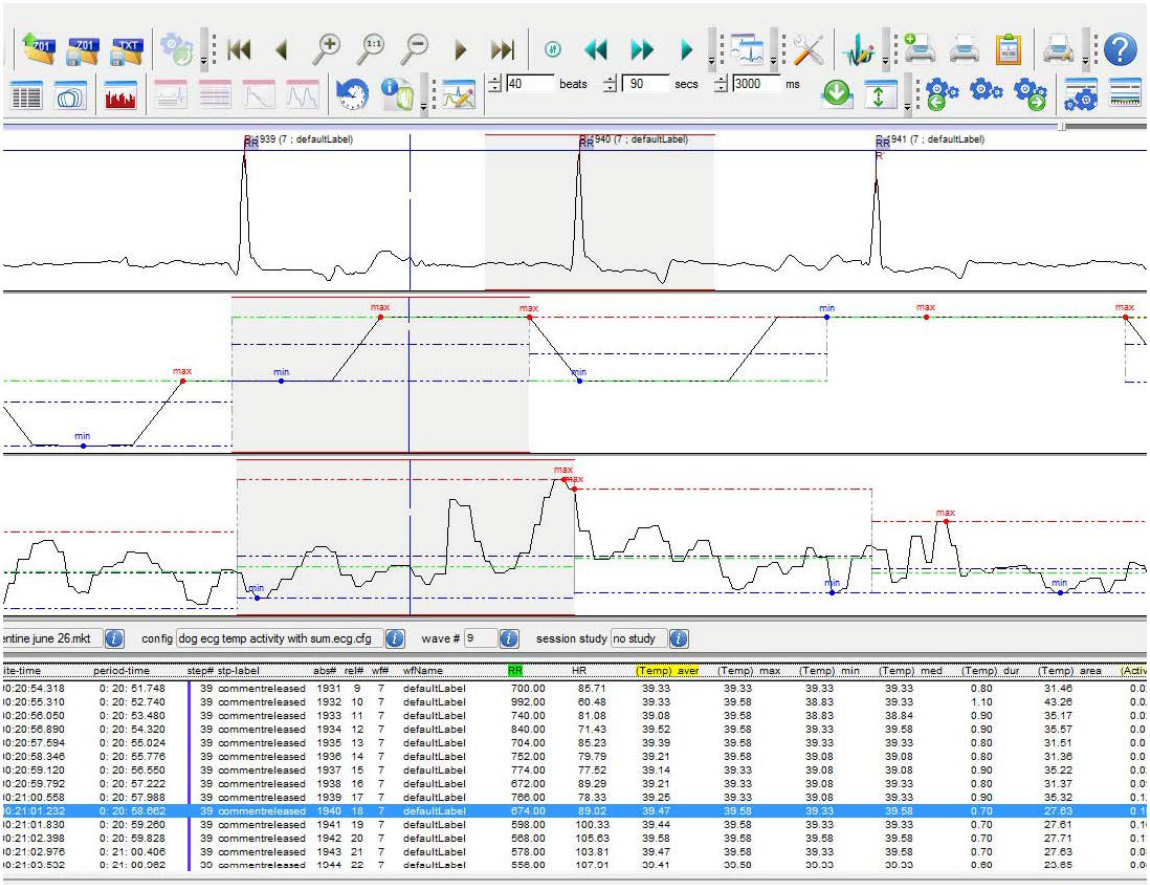
Data analysis

Zones were defined within the open field, and the videos were replayed to determine time spent in each zone, as well as time spent sniffing at an odor port (Figures 30 and 31). Each quadrant containing an odor port measured 9' x 8'6" (7.1 m^2). The central aisle was designated the neutral zone, and measured 6'11" x 16'9" (9.5 m^2). The release zone was along the front wall where the leash release was located, and measured 4' x 24' (8.9 m^2). A dog was considered to have entered a zone when the front half of the dog was in that zone. In the statistical analysis of sniff count and duration, a log transformation was performed on the data because of unequal variances in the raw data. Dogs were categorized as odor-seekers or not based on whether they sniffed the odor ports during the test session. A log transformation was also performed on analysis comparing activity levels during the test session with odor-seeker status.

Figure 31. View of recorded video during playback using Noldus Observer XT software. The dog ('Ace') is shown wearing the telemetry vest and sniffing an active odor port with cat litter.

Analysis of telemetry data was conducted with ecgAuto software. For analysis of the entire session, 30-sec steps were analyzed, beginning when the dog was released through the end of the session. In addition, briefer analyses were conducted in areas of interest such as before and after a recorded comment indicating the dog was sniffing an odor port. A library of ECG waveforms was modified to permit the software to identify similar waveforms from each dog. Temperature and activity data were linked with the corresponding heartbeat (Figure 32). Respiration was analyzed separately by respiratory impedance plethysmography. This produced data on respiratory frequency, breath-breath intervals, tidal volume, flow, and inspiratory and expiratory duration.

Figure 32. Screen shot of analyzed ECG (top), skin temperature (middle), and activity (bottom) traces, with associated data report below. The blue highlighted line in the data report corresponds to the heartbeat highlighted in grey, and the corresponding temperature and activity portions also highlighted in grey.

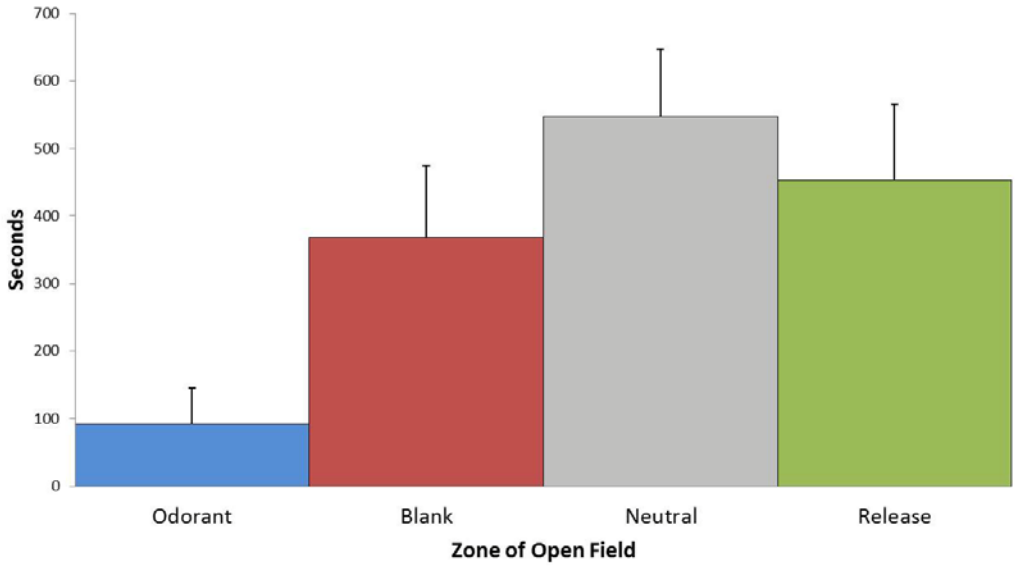


ite-time	period-time	step#	stp-label	abs#	rel#	wf#	wfName	RR	HR	(Temp) aver	(Temp) max	(Temp) min	(Temp) med	(Temp) dur	(Temp) area	(Activ
0:20:54.318	0: 20: 51.748	39	commentreleased	1931	9	7	defaultLabel	700.00	85.71	39.33	39.33	39.33	39.33	0.80	31.46	0.0
0:20:55.310	0: 20: 52.740	39	commentreleased	1932	10	7	defaultLabel	992.00	60.48	39.33	39.58	38.83	39.33	1.10	43.26	0.0
0:20:56.050	0: 20: 53.480	39	commentreleased	1933	11	7	defaultLabel	740.00	81.08	39.08	39.58	38.83	38.84	0.90	35.17	0.0
0:20:56.890	0: 20: 54.320	39	commentreleased	1934	12	7	defaultLabel	840.00	71.43	39.52	39.58	39.33	39.58	0.90	35.57	0.0
0:20:57.594	0: 20: 55.024	39	commentreleased	1935	13	7	defaultLabel	704.00	85.23	39.39	39.58	39.33	39.33	0.80	31.51	0.0
0:20:58.346	0: 20: 55.776	39	commentreleased	1936	14	7	defaultLabel	752.00	79.79	39.21	39.58	39.08	39.08	0.80	31.36	0.0
0:20:59.120	0: 20: 56.550	39	commentreleased	1937	15	7	defaultLabel	774.00	77.52	39.14	39.33	39.08	39.08	0.90	35.22	0.0
0:20:59.792	0: 20: 57.222	39	commentreleased	1938	16	7	defaultLabel	672.00	89.29	39.21	39.33	39.08	39.33	0.80	31.37	0.0
0:21:00.558	0: 20: 57.988	39	commentreleased	1939	17	7	defaultLabel	766.00	78.33	39.25	39.33	39.08	39.33	0.90	35.32	0.1
0:21:01.232	0: 20: 58.662	39	commentreleased	1940	18	7	defaultLabel	674.00	89.02	39.47	39.58	39.33	39.58	0.70	27.63	0.1
0:21:01.830	0: 20: 59.260	39	commentreleased	1941	19	7	defaultLabel	598.00	100.33	39.44	39.58	39.33	39.33	0.70	27.61	0.1
0:21:02.398	0: 20: 59.828	39	commentreleased	1942	20	7	defaultLabel	568.00	105.63	39.58	39.58	39.58	39.58	0.70	27.71	0.1
0:21:02.976	0: 21: 00.406	39	commentreleased	1943	21	7	defaultLabel	578.00	103.81	39.47	39.58	39.33	39.58	0.70	27.63	0.0
0:21:03.532	0: 21: 00.962	39	commentreleased	1944	22	7	defaultLabel	556.00	107.91	39.41	39.58	39.33	39.33	0.60	23.65	0.0

Results

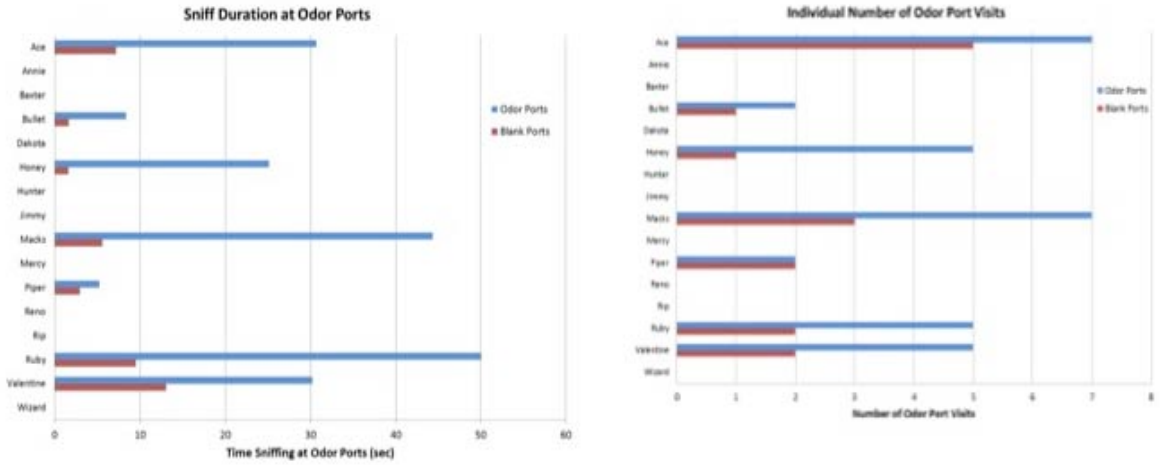
Nine of sixteen dogs spent some time in the zones with odor ports (Figure 33). Many of the dogs rested for extended periods, which greatly influenced the average time for a particular zone. Seven of the dogs interacted directly by sniffing the odor ports (Figure 34).

Figure 33. Mean (± SEM) time spent in the different zones of the odor-cued open field test. This figure is skewed by data for individual animals that were inactive for extended periods in one or more zones.



Dogs that sniffed odor ports made more visits to ports containing odor than the ports which were blank ($p = 0.0246$), and also spent longer times at the ports containing odor ($p = 0.0047$; Figure 34). In addition, the dogs showed more interest in the tuna than the soiled cat litter. Among the seven dogs who actively sniffed odor ports, there were a total of 29 visits to the tuna ports and 4 to the ports containing cat litter. The mean (± SEM) of sniff times at the ports containing tuna, cat litter, and blank were 24.40 ± 7.40, 3.31 ± 2.64, and 2.96 ± 1.28 sec, respectively. The session for one dog (‘Ace’) was terminated after 16 minutes because he knocked down the port containing tuna.

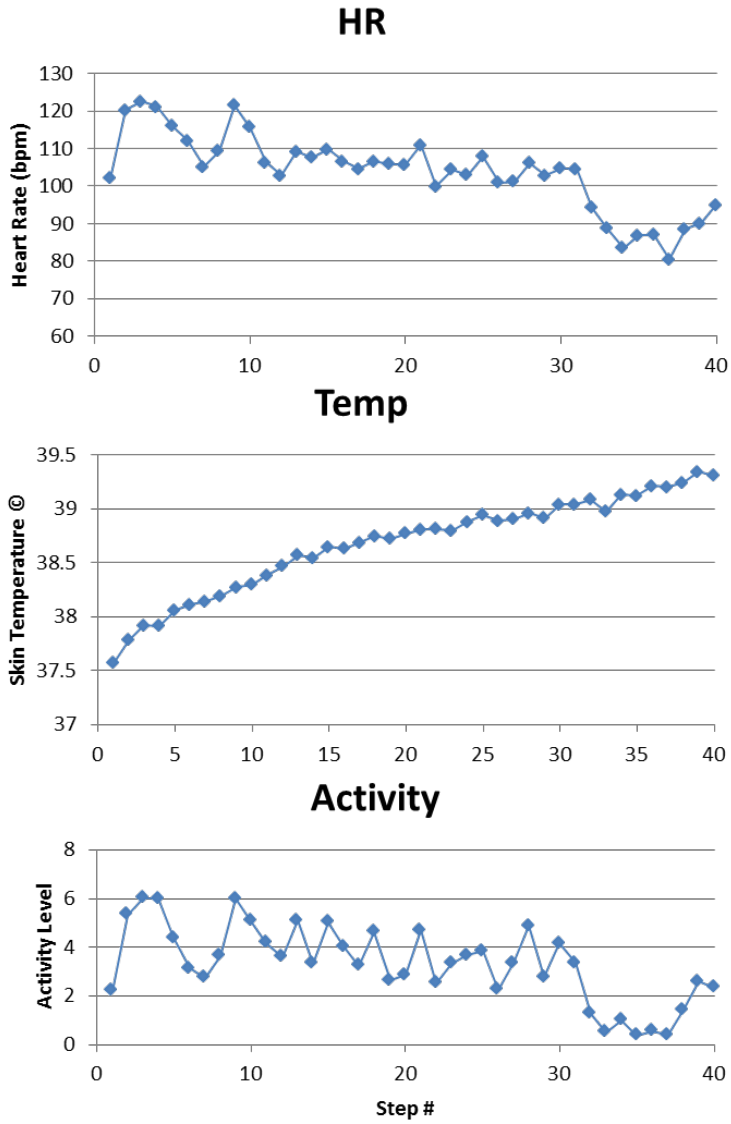
Figure 34. Time shown exploring the odor and blank ports (Left) and total number of odor and blank port visits (Right).



Heart beat length (R-R interval), heart rate, skin temperature, and cumulative activity level for the 20-minute session are presented in Table 22. Average 20-minute heart rates ranged from 63.84 to 127.46 bpm. The corresponding R-R intervals were 943.23 to 473.11 msec. In general, dogs that lay down had decreased heart rates (with longer R-R intervals) during that period. Most, but not all, of the dogs with higher average heart rates were also the ones that were more active. There was a significant correlation between session average heart rate and cumulative activity level ($p = 0.0076$, $r^2 = 0.41$). There was considerable variability in the heart rate during the course of the session, especially in the active dogs (Figure 35). Skin temperature also varied during the course of the test session. In some dogs the temperature rose for most of the session, while in others it decreased. These changes usually appeared to be related to the general activity level of the dog, but may also be related to heating of the electrode by the tape wrapping and the telemetry vest, or to cooling by lying on the concrete floor. The relations between skin temperature and activity, and skin temperature and heart rate were not significant.

Heart rate, temperature, and activity levels for the total 20-minute session were compared with data obtained from the Open Field Anxiety Test (OFT), as described in Phase II. There was not a significant correlation between heart rates obtained following the OFT Day 5 and the session average heart rates in the Canine Olfaction Assessment Test (COAT). Similarly, temperature, as measured rectally in the OFT and as skin temperature in the COAT, was not correlated between the two tests. Distance traveled in the OFT did correlate with the summed activity level measured in the COAT ($p = 0.0153$, $r^2 = 0.35$). The ability to detect fluctuations in these measurements as they occur during a session in relation to certain behaviors, is a valuable tool. The significant correlation between session average heart rate and cumulative activity level in this test is similar to the significant correlation detected between total distance traveled and post-session heart rate on Day 5 of the OFT.

Figure 35. Analyzed 30-sec steps for the duration of a 20-minute recording session for one dog ('Valentine'). A period of decreased activity is seen during steps # 32-38, accompanied by a decrease in heart rate. Skin temperature rose throughout the session.



Respiration was also quite variable during the test session. There were long stretches of rapid, low volume breathing, often seen as panting when the dog was at rest. Active sniffing could be identified by deep, long breaths as seen in Figures 36 and 37. The mean (± SEM) respiration frequency of identified sniffs in Figure 37 was 33.6 ± 6.3 breaths/min, as compared to a rate of 126.2 ± 7.8 breaths/min in the high frequency, low amplitude breaths characterized as resting or panting.

Figure 36. Sample telemetry recordings from one dog ('Valentine'). Top trace is ECG, second is skin temperature, third is activity, and bottom trace is respiration. The dog was resting for approximately 2/3 of this 90-sec tracing, then got up and started sniffing, seen in the burst of activity and the large, deep breaths on the right side of the figure. Inset on the bottom right (Rabbit sniff) shows similar sniff pattern seen in rabbits in response to a test odorant (Grajski et al., 1989).

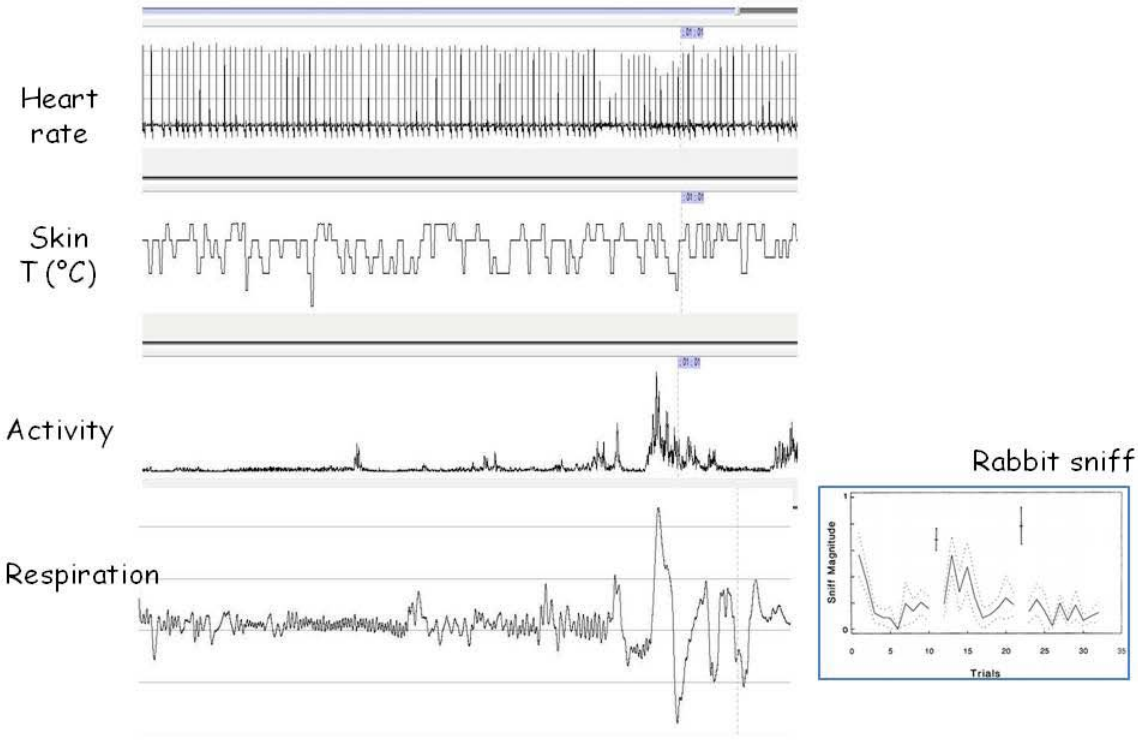
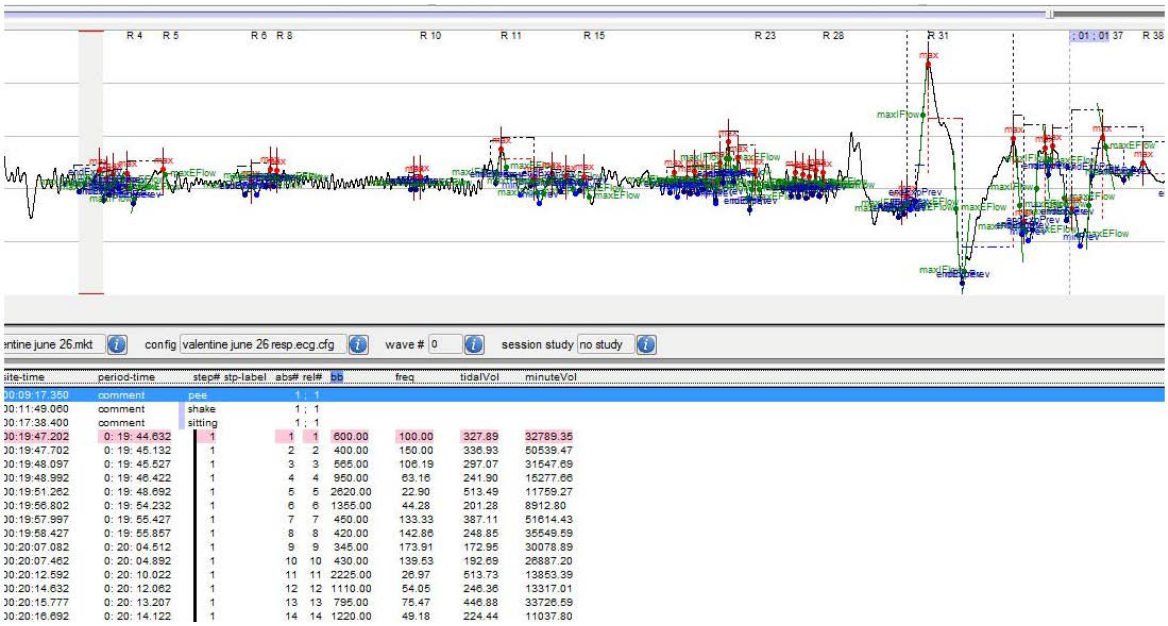


Figure 37. Screen image showing analysis of a 90-second segment of respiration, corresponding to lower trace of previous Figure 36.



The dogs were categorized as odor-seekers or not based on whether they sniffed any of the odor ports during the session. Seven out of 16 dogs were categorized as odor-seekers. Total activity during the session was highly correlated with odor-seeker status ($p = 0.0001$, $r^2 = 0.63$). Odor seeker status was not statistically related to sex, coat color, prior imprint odor training at K2, or anxiety phenotype ('worst'/'non-worst') from the previous open field test (Phase II). DNMP and olfactory discrimination (vanillin or AN) performance did not predict odor-seeker status (data not shown). This result is similar to the observation that DNMP performance was not predictive of performance on olfactory discrimination learning. Odor-seeker status was not influenced by ERT scores or the anxiety score from the OFT.

Discussion

Various methods have been employed in order to measure the external airflow sniff dynamics in humans. These include the use of (a) respiratory belts placed around the abdomen, chest, or both that measure respiratory sniff airflow–related expansion and contraction, (b) thermistors placed at the nares that measure the sniff airflow–related changes in temperature, (c) cannulas places at the nares and linked to pressure sensors that measure sniff airflow–related changes in relative pressure, and (d) pneumotachometers placed at the inlet of a nasal mask where they similarly measure sniff airflow–related changes in relative pressure (Johnson et al., 2006). These methods are not all equally effective in measuring sniffing within an olfactory task. At times, the only information necessary is whether the subjects sniffed and when. For such gross information, it is likely that any of these measures is sufficient (Johnson et al., 2006). We chose to use respiratory belts in our canine subjects. This approach allowed us to collect data while the dogs were ambulating freely.

In our experiment we were able to use remote telemetry to identify times when dogs were actively searching and sniffing for an odorant source. Waveforms seen during active sniffing are similar to those reported for people and animals. The ability to detect sniffing behavior is

important since olfaction typically consists of both sniffing (airflow in the nostril regardless of odor presence) and smelling (the percept of odor regardless of airflow in the nostril). Although olfactory perception is usually assumed to reflect the latter, it is largely dependent on the former. We were able to discriminate odor-cued behaviors from non-odor cued events. For example animals engaged at an active odor port had a higher frequency of sniffing compared to other locations.

The methods we have developed can be applied in many ways. For example, the relationship between airflow and nasal cavity anatomy is well understood. Indeed computational fluid dynamic (CFD) models generated from anatomically accurate computed tomography, magnetic resonance imaging studies, or serial histologic step sections are available for multiple species including humans, rodents, macaque monkeys, and dogs (Craven et al., 2009; Longest et al., 2012). Measurement of the respiratory dynamics of free roaming dogs while performing an odor-based behavioral task can generate respiratory physiology data that further calibrates these CFD models. Subsequently, the models could be used to determine respiratory breathing patterns that could sufficiently alter airflow dynamics resulting in degradation of olfactory performance. Conceptually similar approaches have been used in toxicology and other disciplines (Schroeter et al., 2008).

Another objective of this study was to evaluate whether wearing a telemetry vest altered the normal activity of dogs. We found a strong statistically significant association between the telemetry-acquired activity of dogs during the COAT and distance traveled during our previous OFT. This finding suggests that dogs that were active during our initial OFT experiment were also active during the COAT session. Qualitatively we could not appreciate an increase in anxious behaviors while the dogs wore the telemetry system. These findings likely reflect the way we slowly acclimated the animals to wearing the jackets. One significant advantage to the telemetry jackets is the ability to collect relevant physiology data in real time. This ability may help to refine the OFT we developed during an earlier project (Phase II). Future experiments directly evaluating distance traveled and telemetry-acquired activity measurements along with assessment of the dog's behavior during an OFT will help further determine whether wearing the telemetry jacket changes a dog's behavior.

The present experiment also sought to determine whether the COAT could serve as a screening tool for behaviors critical to IDD function. The use of a remote release without human intervention allowed us to assess the dogs': (a) eagerness to search, (b) ability to focus on searching while ignoring distracting stimuli, (c) ability to work effectively in a novel situation; and (d) motivation for sniffing. The odor cues that we used included soiled cat bedding (that contained urine and fecal material) and tuna oil. We used static odor ports for odor delivery. One reason for using a static odor presentation system was to make the odor detection task more challenging. Doing so allowed us to identify animals that would respond to relatively low air concentrations of the test odorant (a crude test of olfactory acuity). As expected, the dog's natural tendency to roam during the COAT was highly predictive of their ability to detect odor (search motivation). The exploratory and/or odor-seeking behavior of dogs in the COAT represents an integration of multiple behavioral and cognitive domains.

In many ways, the COAT is similar to indoor environments used to train IDDs and other detector dogs. Importantly, the COAT differs in some ways. Since the dog is not under the control of a handler its performance in the COAT is under more allocentric control. In essence, exploring the novel environment and finding odor serves as its own reward. The results of the COAT allowed us to segregate dogs into several distinct groups: active/odor seeking; active non-odor seeking; and inactive non-odor seeking dogs. We believe that the last group may represent a

subpopulation of dogs that may be less suitable for IDD work. Additional studies will be needed to confirm this observation.

We also examined whether performance on the COAT correlated with other measures of cognitive function or olfactory discrimination. The behavioral platform we used to assess these measures was a derivative of the Wisconsin General Test Apparatus and requires extensive behavior shaping. The operant system we used to evaluate cognition and olfaction in dogs was also highly dependent upon the animal's motivation to receive a food reward.

Several refinements to our COAT could improve the utility of this test. Refinements could include the ability to measure the distance moved by an animal during a test session. Our earlier work also documented the value of assessing whether an animal is demonstrating stress or anxiety responses in the COAT arena. Importantly the dogs' odor seeking behavior and general activity were not correlated with the dog's previously determined anxiety phenotype.

SECTION III: FIELD STUDIES CONDUCTED AT K2

PHASE VIII. THE ROLE OF OLFACTORY PRIMING ON THE DETECTION OF C4

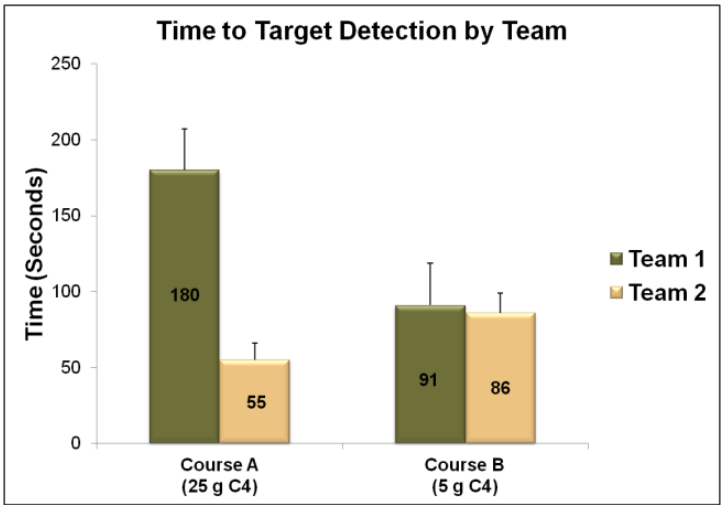
Background

A number of agents used as explosives are used to train IDDs to detect relevant odors, a process called imprint training. One of the agents used for imprint training is C4, which is a common form of the plastic explosive known as Composition C. C4 is particularly suitable for use as a test agent, since it is stable and insensitive to routine physical handling. In addition, the olfactory information released is a function of the amount of C4 by weight. Thus, a larger amount of C4 by weight will produce a larger olfactory signature than a smaller amount of C4. This simple relationship provides a means of measuring olfactory acuity in candidate IDDs.

Our first experiment was designed to evaluate whether days of training of candidate IDDs influenced their ability to detect an odorant (C4) in an open field. We hypothesized that dogs with more days of odor training would detect a given amount of C4 more quickly than dogs with fewer days of odor training. In this experiment, two equal sized teams of dogs (n = 8 dogs/team) were tested. Dogs in Team 1 had less than 45 days of odor training and were approximately midway through their odor training at K2. Dogs in Team 2 had approximately 100 days of odor training and were nearing the end of their training at K2. All dogs were trained on > 100 g of C4 and had been taught to 'cover' (lie down in sternal recumbency) in proximity to the odor source. All dogs had mastered the cover behavior in response to the presence of C4. Our experimental outdoor test field was approximately 20 m x 90 m in size. Samples of C4 (5 or 25 g) were placed approximately 60 m from the start point. The samples were placed in a small (2 cm) "dig," a depression in the ground, then covered with an inconspicuous layer of leaves, sticks, and other vegetative debris. Five additional depressions were prepared similarly and served as false digs. Dogs in both groups were given familiar verbal commands by their K2 trainers (e.g., "hunt it up", "go find it") to begin the search. The time to detect the C4, as well as true positive and false negative odor covers were recorded. Odor searches occurred in the following order: 25 g of C4, 1 hour rest, 5 g of C4. In each case the time from release to positive and negative cover, and the cover rates were recorded by a trained observer. False cover rates were rare and not related to days of training (data not shown).

Figure 38 shows the results of this experiment. Dogs in Team 1, with fewer days of odor training, took more time to find the larger amount (25 g) of C4 than their more trained counterparts in Team 2. We anticipated that this difference in days of training would be magnified if the search involved less C4 (5 g). This proved not to be the case. During the second search both groups of dogs performed equally well, detecting the smaller amount (5 g) of C4 in the same amount of time required by the more experienced dogs searching for the larger amount (25 g) of C4. This experiment prompted our desire to evaluate whether olfactory priming with C4 could improve the ability of dogs to detect C4.

Figure 38. Mean (± SEM) time to detect 25 g, but not 5 g, of C4 is influenced by the length of odor imprinting training ($p < 0.05$). Team 1 had < 45 days of training while Team 2 had approximately 100 days of odor training.



The neural phenomenon of priming can be described as the influence a previously encountered stimulus (the prime) has on the processing of a second stimulus (the target). The target can be either identical to the prime or related in some respect to the prime. It is usually assumed that priming rests on a 'spread of activation' process in the central nervous system (McNamara, 1992). When the target stimulus is perceived, the residual activation serves as a memory trace that facilitates (i.e. 'primes') the processing of the target. In this way, the target stimulus is detected more quickly than it would have been without precedent processing of the prime.

Although most of the critical work on priming has been carried out using visual and auditory modalities—where stimuli are relatively easy to control—olfactory priming and beneficial effects on olfaction have been observed (Koenig et al., 2000). Operationally, an olfactory prime is often referred to as "pre-scent." The goal of the following experiment was to determine whether short-term exposure of a dog to an odor ("the prime") could enhance subsequent ("target") odor detection. We tested this paradigm in field tests conducted at K2. The "prime" was a short exposure to C4 in dogs already trained to this odorant. The "target" was C4 exposure in a test-field situation. The experiment was repeated on the same dogs after 84 days without exposure to C4.

Materials and Methods

Animals

Dogs used for this study were 12 NCSU dogs housed at K2 (cohort 2). All dogs used in this experiment had been previously trained (i.e. imprinted) by K2 staff to detect and "cover" on approximately 100 g of C4. All dogs were trained to cover on a variety of test odorants including C4. Dogs were last imprinted on C4 3 days before the first trial. The test field had two components (Figure 39), the odor lane and the test field.

Odor lane

A 2.4 X 31 m linear outdoor "odor lane" was used for training dogs to detect odors and to "prime" the dogs for assessment in the test field. The odor lane had numerous PVC pipes placed

below ground (the tips extend 1-3 cm above the surface of the soil), which allowed odorant to be placed in one or more locations. For this experiment, one location was utilized and dogs performed individually. Dogs in Group 1 (n = 6) initially explored the odor lane while C4 was present (pre-scent; "prime"). Dogs in Group 2 (n = 6) performed a negative search (blank) of the odor lane.

Test field

Shortly after completion of the pre-scent search (≤ 5 minutes later) the dogs were individually directed to search a large (54 x 110 m) field where 25 g of C4 was hidden near a barrel 77 m from the start location. The time required to detect the C4, as well as the number of true positive and false negative odor covers were recorded. Once Trial 1 was completed, imprinting training with C4 was stopped until Trial 2 was completed. The two trials were conducted 84 days apart.

Results

Individual dog data is presented in Table 23. The overall mean values are presented in Figure 40. In trial 1 (conducted while actively being trained), the mean (± SEM) time to detect C4 in the animals primed with or without C4 was 152.2 ± 31.4 and 113.7 ± 26.4 sec, respectively (NS). In trial 2 (conducted approximately 90 days after cessation of training), the mean (± SEM) time to detect C4 in the animals primed with or without C4 was 103.2 ± 24.9 and 45.8 ± 12.8sec, respectively (p = 0.0869; excludes data from 1 dog during Trial 2 – noted to become distracted by an extraneous sound during search) The time to detect C4 was not affected by the nearly 90-day stop in odor imprinting/training (Figure 40). We also found that the dog's NCSU ERT score was not correlated with the animal's time to detect the test odorant (data not shown).

Figure 39. Schematic description of the outdoor "odor lane" (Left) and test field (Right). Drawing is not to scale.

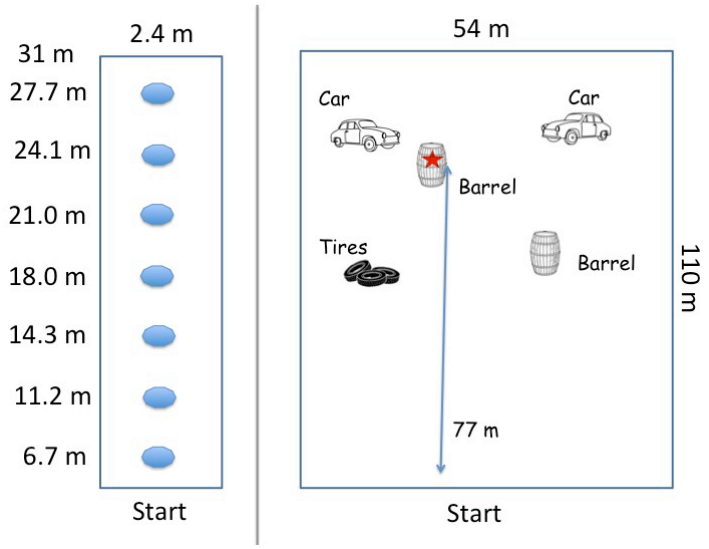
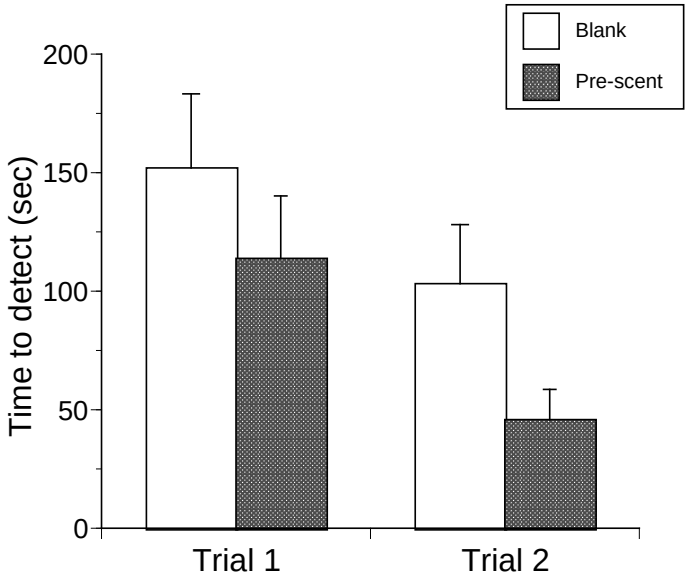


Figure 40. Time to detect C4 in the K2 outdoor training facility. The location of the C4 was held constant during the test session. Pre-scenting consisted of a single search using the K2 outdoor odor lane – dogs were either provided with C4 in the lane (pre-scented) or not (blank). The dogs were then immediately asked to search the large K2 test area for the presence of C4 – with time to detect C4 being the experimental criterion examined.



Discussion

Odors are more difficult to manipulate than visual or auditory stimuli, and the first aim of the present experiment was to investigate whether a priming effect could be elicited with an odor, in this case C4. In general, we observed that dogs responded faster in the experimental test field after a brief encounter with the test odor (C4). Although the effect did not reach statistical significance in either trial ($p = 0.0869$ in trial 2) the trend was consistent between experiments. This finding may suggest that the use of odor training aids before a search could be of benefit. This advantage must be weighed against the problems associated with carrying and using odor-training aids in the field.

The beneficial effects of odor priming can have beneficial effects that extend beyond a specific olfactory task. In people, odor priming has been shown to positively affect memory, vigilance, pain perception, self-perception/confidence, and alertness (Johnson, 2011; Moss et al., 2003). It is uncertain whether similar responses would be seen in dogs. An important finding that arose from this experiment is that a 90-day suspension of C4 odor training did not adversely affect the performance of dogs on detecting this explosive. This is not an implausible finding, given the remarkable capability dogs have exhibited for remembering odors over long periods of time. It has been reported that dogs can retain high levels of detection performance after a period of at least 4 months without odor training, and possibly longer (Johnston, 1999). Future research investigating the limits of memory duration and capacity in dogs would be useful in the training of IDDs. Extrapolating these results to deployment situations, we suggest that dogs are "primed," i.e. exposed for short periods of time to relevant odors in order to optimize their capacity for detection of explosives.

PHASE IX. SOIL DEPTH AND ITS IMPACT ON ODOR DETECTION IN DOGS

Background

Improvised explosive devices (IEDs) represent an important threat to U.S. military and civilian personnel deployed to Afghanistan and other hostile areas. IEDs are relatively simple, low-tech devices, which routinely use command wire, pressure plates, or radio-controlled triggers. Agricultural fertilizers are often used for their oxidant properties and the fact that fertilizers contain ammonium nitrate (AN). In fact, many fertilizer-based IEDs contain between 10 to 25 kg or more of AN. When combined with other elements, they comprise many IEDs found in Afghanistan.

Scent canines are used by many government and law enforcement agencies as a detection device. IED detector dogs (IDDs) and their U.S. Marine Corps (USMC) handlers are an important counter-IED system in hostile environments. These dogs are trained in the U.S.in a USMC-approved training program to detect a number of explosives of interest (AN, C4, detonating cord, among others) and to "cover" (lie down) to signal to their handler when an odor is detected. Each IDD works off leash and can examine large areas of ground during its search. The ease with which IDDs can be trained and the dogs' willingness to cooperate with humans are crucial for successful IED detection.

Of critical importance is the ability of IDDs to detect and recognize the odors associated with explosives. Even minute amounts of a particular odorant may be detected and recognized due to the extraordinary sensitivity of the dog's nose. The initial process of odor discrimination begins in the olfactory neuroepithelium located in the nasal cavity. Odorants activate olfactory receptors on the cell surface of an olfactory neuron that initiate further signal transduction to the brain (Firestein 2001). Odor intensity varies with the concentration of the odorant in the air (Lapid et al., 2009). This may be the result of higher concentrations of odorants reaching the nasal epithelium, activation of a larger number of receptors, or recruitment of the trigeminal system in odorant detection (Frasnelli et al., 2011). The bottom line for canine olfaction is that lower air concentrations of a material can reduce the ability of a dog to detect it.

This relationship between air concentration and odor detection may have important implications for buried materials where odorant concentrations may be reduced. For example, odorants may undergo solid- or liquid-phase reactions with the soil, they may react or adsorb to soil particles, soil can serve as a diffusion barrier for the release of materials. In dry, sandy environments such as Afghanistan, the low soil moisture decreases the availability of explosive molecules in vapor (because of increased vapor adsorption to the soil). Odorant molecules may either leak or permeate through the soil.

Some studies, have demonstrated that dogs were of limited use in detecting buried mines (Ashton and Eayrs, 1970) while others provide strong positive evidence (Nolan and Gravitte, 1977). Canines trained to detect human remains are capable of detecting extremely small (e.g., teeth) or aged scent sources that are often buried. Canine performances as a scent detector can be affected by training, familiarity with the scent source, and environmental conditions (Cablk and Sagebiel, 2011; Komar 2009, Oesterhelweg et al., 2008). Little information is available regarding the capability of IDDs to detect buried explosives. For the most part, candidate IDDs are trained using surface odor training aids. Our goal in this experiment was to evaluate the effectiveness of trained Labrador Retrievers at detecting buried AN.

Materials and Methods

Animals

NCSU dogs used for this study were housed at K2 (cohort 2). All dogs used in this experiment had been previously successfully trained by K2 staff to detect AN and C4 hidden above ground or in shallow depressions in the ground, covered with leaf litter. Dogs were trained to detect 8 ounces (227 g) to 3 pounds (1361 g) of AN. Dogs were generally trained to detect 250 g of C4. In response to detection of a variety of test odorants including C4 and AN, all dogs were trained to cover (Figure 41). Dogs were given verbal commands by their K2 trainers (e.g., "hunt it up", "go find it") to begin all searches. Climatic conditions were recorded for each test session.

Figure 41. A dog from cohort 2 demonstrated the behavior known as 'cover' upon detecting an odor source. The sparse vegetation present in the test fields is also shown.

Section 1. Ability to cover

The first set of trials were designed to confirm that dogs would cover reliably on AN. In this trial, the dog's cover behavior was assessed by placing known quantities of AN (ranging from 0.25 to 250 g) approximately 30 m from the start point. AN was pre-weighed and placed into a nylon mesh bag for containment. Placement of AN was randomized during the study. The test field consisted of 5 buried 61 cm x 10.2 cm diameter pieces of PVC pipe in the ground. The pipes were spaced approximately 15 feet apart and arranged with 1 pipe at each corner of a square with the last in the middle. The entire field was surrounded by a barrier fence, designed to keep the dogs contained within the small area. Into one pipe was placed a 7.6 cm diameter PVC pipe containing a sample of AN, either at the surface or buried under 7.6 cm of soil. The rest of the pipes contained identical 7.6 cm diameter PVC pipes containing only soil and the same mesh that held the sample. In these trials, the dog's cover behavior was assessed by a K2 trainer. For each trial the dogs were scored as having either covered on odor (1) or not (0). False covers were scored as a '0'. At least 3 trials were completed for each AN amount. The % correct trials were calculated for each dog (# correct covers/# trials, where 12 trials were used). Small quantities of AN were used in these trials to assess whether differences in olfactory thresholds were present.

Section 2. Timed trials with surface AN

The next phase of the work was designed to determine the time required for each dog to detect surface AN. We used an approximately 40 m x 40 m field at the K2 facility. The experiment was conducted over several days using a common starting point. The test field was relatively flat and surrounding trees and other landscape elements helped to define the boundaries of the test area. The test field consisted of sandy soil and sparse vegetation. The bag containing the odor was randomly placed under vegetation that was already present, approximately 35 m from the start point. Additional swatches of the mesh were placed in random locations around the field in the same manner as the bags containing the odor. Trials performed on a given day began from the same start point. Known quantities of AN (0.25, 2.5, 25, and 250 g) were placed at a location approximately 35 m from the start. One quantity of AN was used for each trial (n = 1 trial/AN sample; 4 samples per dog). The AN samples were placed in a small (2 cm) depression and covered with leaves, sticks, and other vegetative debris. Several additional depressions were prepared similarly (minus AN) and served as false digs. Nine dogs were used for this phase. Three dogs ('Hannah', 'Harley', and 'Twiggy') were excluded due to their unreliability in indicating a positive find of AN during Section 1. The time required to detect the AN, as well as the number of true positive and false negative odor covers were recorded. Dogs had a minimum of 45 minutes to rest between trials.

Section 3. Timed trials with surface AN: Behavior generalization

A different approximately 40 m x 40 m field at the K2 training facility was used for this study. Known quantities of AN (25 or 250 g) or C4 (25 g) were placed at the surface with additional disturbances created. A single trial with 2.5 g of AN was also used; however, dogs were unable to find this quantity and, therefore, additional trials with this quantity were aborted. The C4 or AN samples were placed in a small (2cm) depression and covered with leaves, sticks, and other vegetative debris. Several additional depressions were prepared similarly (minus AN or C4) and served as false digs. All trials performed on the same day began from a common start point. Dogs were given verbal commands by their K2 trainers (e.g., "hunt it up", "go find it") to begin the all searches. Seven dogs were used for this phase. Two dogs ('Charlie' and 'Cricket') were excluded because they were in heat. The time required to detect the AN or C4, as well as the number of true positive and false negative odor covers were recorded. Dogs had a minimum of 45 minutes to rest between trials. Wind direction was recorded, but not found to have any real impact until the dog was within 1.2 m of the odor.

Section 4. Buried AN trials

This phase of the work was completed initially using a smaller semi-enclosed test area for these experiments. The test area measured 7.62 x 7.62 m and the border of the test arena was defined using a commercially available 1 m tall silt fence. The test area was equipped with several 0.61 m holes that contained a 0.61 m long piece of 10.2 cm PVC pipe. The 10.2 cm pipe served as a container for a second smaller (7.6 cm) diameter PVC pipe that was enclosed on the bottom. The inner pipe allowed us to place known quantities of AN under a fixed soil depth. The pipe system provided the dogs with a visual cue that was similar to that found in the outdoor odor lane (Phase VIII). Unprocessed native sandy soil obtained from the K2 facility was used for this experiment. Pre-weighed quantities of AN were used (0.25, 2.5, 25, and 250 g of AN). The AN was allowed to permeate the tube for at least 30 minutes prior to the start of the experiment. The dog's ability to cover on odor was assessed by the K2 handler. For each trial the dogs were scored as having either covered on odor (1) or not (0). False covers were scored as a '0'. At least 4 trials were completed for each AN amount. The % correct trials were calculated for each dog.

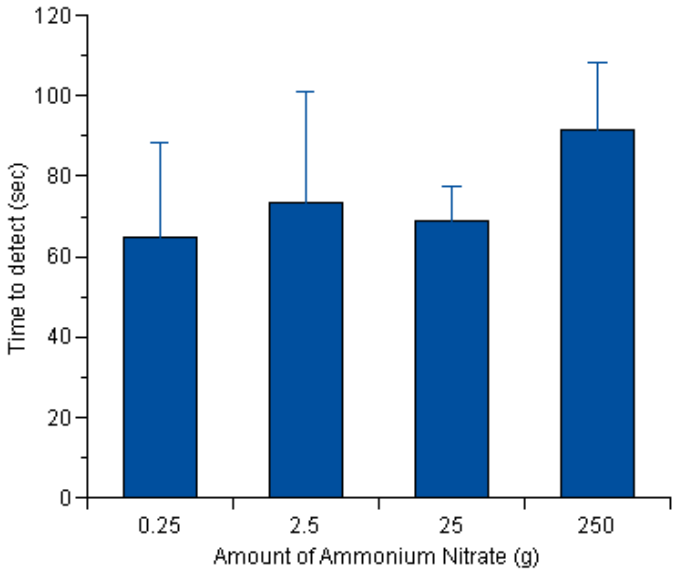
We also assessed the ability of dogs to detect AN that was buried in a larger (40 x 40 m test field). In this experiment known quantities of AN were buried 7.62 cm below the surface of the top soil layer. The AN samples were placed in a nylon mesh bag, buried under the soil, and the small disturbance was covered with leaves, sticks, and other vegetative debris. Several additional negative disturbances were prepared similarly (nylon mesh minus AN) and served as false digs. All trials performed on the same day began from a common start point. Dogs were given verbal commands by their K2 trainers (e.g., "hunt it up", "go find it") to begin the all searches. Seven dogs were used for this phase. Two dogs ('Charlie' and 'Cricket') were excluded because they were in heat. The time required to detect the AN, as well as the number of true positive and false negative odor covers were recorded. Dogs had a minimum of 45 minutes to rest between trials.

Results

Ability to cover: The ability of dogs to cover on known quantities of AN (ranging from 0.25 to 250 g) is presented in Table 24. Cover behavior was not significantly affected by either the amount of AN used ($p = 0.771$; all dogs or $p = 0.846$; final cohort) or NCSU ERT score. Based on these findings we excluded 'Twiggy' and 'Hannah' because of consistently poor performance (cover efficiency < 50%). We also excluded 'Harley' because of a lack of hunting drive.

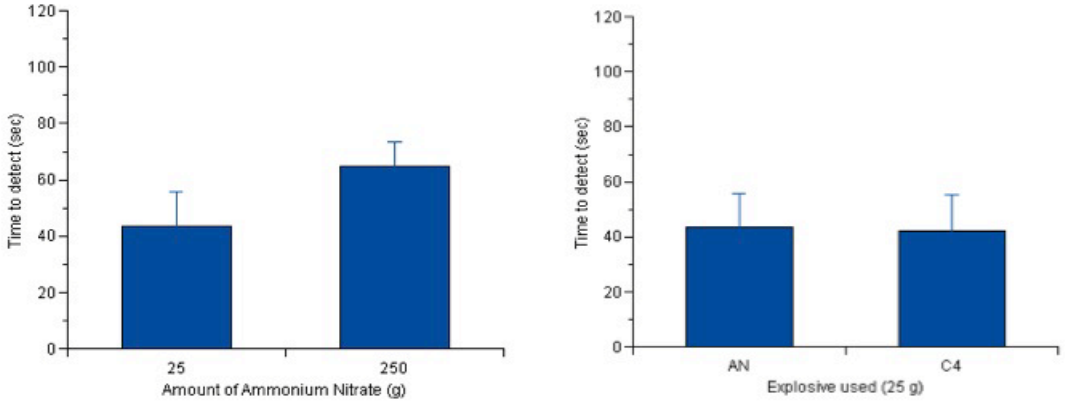
Timed trials with surface AN: Results from our initial trials with surface AN are presented in Figure 42. The time to detect AN was independent of the amount of AN used ($p = 0.6761$). No effect on false cover frequency was seen.

Figure 42. Mean (± SEM) time required to detect surface AN by Labrador Retrievers. The quantity of AN used (0.25 to 250 g AN) did not influence the time required to detect AN.



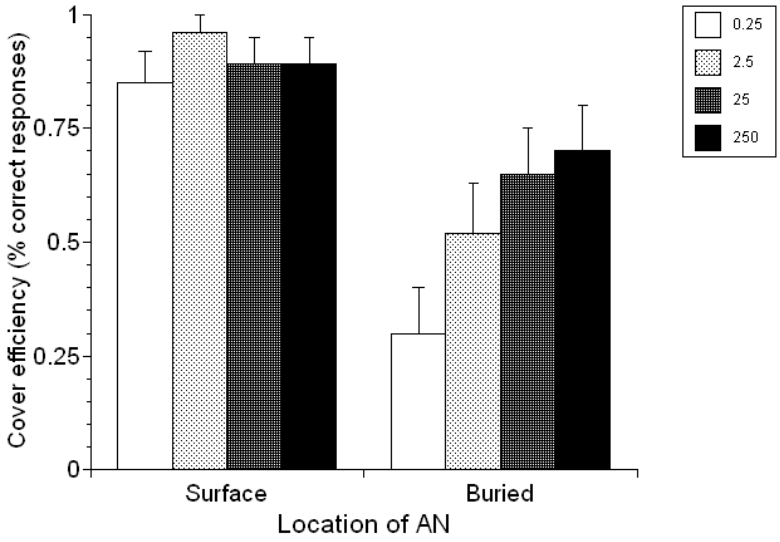
Results from our next series of trials with surface AN are presented in Figure 43. Like our previous experiment, the time to detect AN was independent of the quantity of AN used ($p = 0.167$). No effect on false cover frequency was seen. Likewise, ERT score and mean time to detect AN (pooled across trials using 2.5 to 250 g AN) was not correlated. We did not observe a statistically significant difference between the times needed to detect 25 g of either C4 or AN (Figure 43). Summary data for the dogs is presented in Table 25.

Figure 43**.** Mean (± SEM) time to detect either 25 or 250 g of AN (Left) or 25 g of either AN or C4 (Right). Data shown were collected on the same test day under similar environmental conditions.



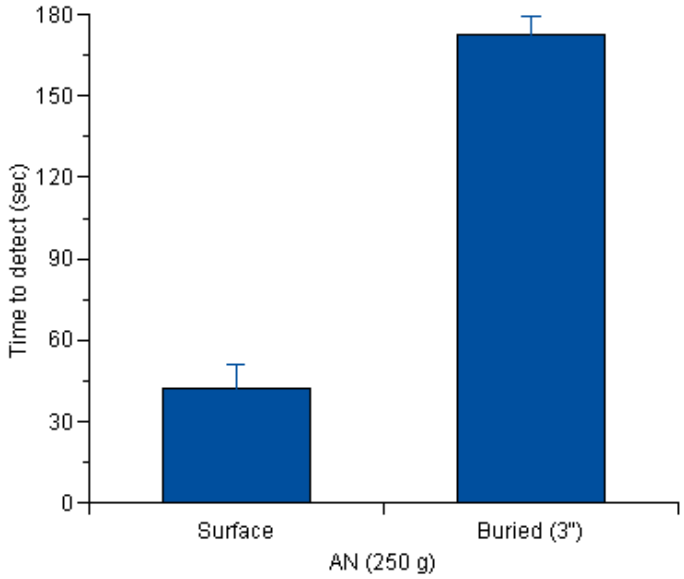
Ability to detect buried AN: The ability of dogs to cover on buried AN was more variable when compared with the original study using material on the surface. The ability of a dog to cover on buried odor was marginally affected by the amount of AN ($p = 0.0579$). This result was in contrast to our study with surface AN where no effect of quantity was seen. Detection of AN was significantly decreased when the AN was buried ($p = 0.004$; Figure 44; when compared with trials conducted with surface AN).

Figure 44. Mean (± SEM) positive cover rates seen with varying amounts of AN presented to dogs at either the ground surface (Left) or under 15 cm of soil (Right). Quantities of AN used ranged from 0.25 to 250 g.



Our final set of experiments evaluated whether dogs would detect buried AN in an open field environment. As expected, the time to detect was longer (Figure 45) with buried material ($p < 0.0001$). In most cases (6/7 dogs) the dog was unable to detect 250 g of AN that was buried 8 cm below the surface.

Figure 45. Mean (± SEM) time to detect 250 g of AN when presented to dogs at either the ground surface or under 8 cm of soil.



Discussion

The results of our experiments yielded several new insights into the odor detection capabilities of Labrador Retrievers. First, dogs that have been imprinted onto different odors may not reliably demonstrate the behavioral change (e.g., cover) used to signal the presence of an odor of concern. In our study we found that approximately 25% of the dogs available to us proved unreliable at demonstrating the desired cover behavior in response to the presence of AN. The finding of variable signal detection behavior (e.g., cover) in dogs should be an exclusion criterion for candidate IDDs. The methods we used in our studies can be easily adapted for this purpose. Inconsistent or poor cover behavior was not correlated with the dog's NCSU ERT score, a measure of emotional resilience and reactivity in dogs. This observation is similar to what we saw in more controlled laboratory studies of olfactory discrimination in dogs (Phase V). The cohort we used was unbalanced with respect to sex or coat color so the influence of these physical characteristics could not be examined in this study.

We found that those dogs that demonstrated reliable cover behavior were consistent in their ability to detect AN or C4. Under the conditions of our test, the time required for the dog to detect AN was largely independent of the quantity of AN used. Observation of off lead working dogs was quite revealing and showed that dogs developed a search pattern that quickly brought them to the odor source. When all the trials are considered together we found that dog generally found the source of odor in less than 1 minute irrespective of the type of material (AN or C4) or quantity of material. We found that dogs could reliably find surface quantities of AN as small as 2.5 g. The ability to detect 0.25 g of material in an approximate 1600 m^2 search area represents a functional "odor threshold" for this material. The time required to detect C4 or AN was remarkably stable across multiple days even though ambient air temperature and wind velocity varied considerably.

Our results with buried AN were more mixed. We used two different methods to present "buried" AN to the dogs. In one series of experiments, the AN was contained within a PVC pipe system that allowed us to vary the soil depth under investigation. The top of the PVC pipe system was either at or slightly above the surface of the ground. This design mimicked an outdoor 'odor lane' that is used at K2 for imprinting and other odor work. We found that when this system was used, dogs could generally detect 'buried' AN. For other experiments we buried the AN material in a large open field. These studies often used 250 g of AN buried under 8 cm of soil. The dogs used in this experiment showed that they could find this quantity of odor when presented to them in the PVC pipe system where visual cues were also present. We found that the ability of dogs to detect AN in an open field was largely abolished when the AN was buried under 8 cm of soil.

Our findings with buried AN may have important implications for the training of candidate IDDs. First, the dog-trainer interaction must be considered. Trainers must remain vigilant to minimize visual and other cues to dogs engaged in an active search. This can be difficult since dogs have demonstrated a remarkable ability to detect cues from human beings (Reid, 2008). Likewise, it becomes critical that test areas and reward locations be varied during training to minimize dogs associating certain areas with rewards. Although this behavior was not under direct investigation we did observe this behavior when we worked dogs in a test field used for training dogs to search vehicles. In this occurrence, dogs consistently went to a location known to be rewarded (i.e., the car) even though the odor source was distant from that site. The size of the test field and the distance of the odorant source to the start location was comparable to that used previously with our dogs. We also produced evidence that the ability of dogs to detect buried odor is influenced by the context in which the trial occurs. We found that the dogs likely use a combination of visual and olfactory cues to detect buried AN. Training protocols for dogs should be varied to exercise these cognitive abilities of dogs.

Our work is not intended to mimic operational use of IDDs. The amount of AN that we used is quite small and was designed to allow us to develop a relative olfactory threshold for this material. Although dogs in our experiment could not detect AN buried in a large field, this result should not be construed to mean that dogs can not detect buried IDDs in Afghanistan or other places. The operational experience of the USMC confirms that this is indeed the case (i.e., IDDs can find buried AN). Our experiments were designed to test the limits of certain training practices used by K2 (and other organizations).

PHASE X. PILOT STUDIES EXAMINING PROTON PUMP INHIBITOR EFFECTS ON CANINE OLFACTION

Background

Endurance canine athletes have a markedly increased incidence of gastrointestinal disease compared to less athletic counterparts (Davis et al., 2003). Hallmarks of exercise-induced gastrointestinal disease in dogs include gastritis, diarrhea, gastric erosion, and gastric ulcers. Gastric mucosal lesions occurred in 5 out of 6 Labrador Retrievers undergoing a mock deployment exercise (personal communication, MS Davis). Oral administration of the proton pump inhibitor omeprazole (1 mg/kg/day, e.g., Prilosec) has been successfully used to manage exercise-induced gastritis and gastric ulcers in dogs (Davis et al., 2003, Williamson et al., 2007, 2010). The prophylactic use of omeprazole to reduce the incidence and/or severity of exercise-induced gastrointestinal disease in IDDs is currently under consideration.

Before initiating the prophylactic use of omeprazole it is important to consider whether this drug may adversely affect olfaction in IDDs. Collection and histologic evaluation of the nasal tissues and the olfactory bulb is considered optional in drug safety testing by the Organisation for Economic Co-operation and Development (OECD, 2008). The US Food and Drug Administration (US FDA) uses a similar approach. Reports of recent experience with Zicam nasal products (e.g., Zicam Cold Remedy and Zicam Cold Remedy Swabs) provide one example in which nasal toxicity occurred as a result of drug administration. These products contained zinc acetate and zinc gluconate and were associated with a high incidence (over 100 cases were reported to the US FDA) of self-reporting of anosmia (loss of sense of smell) following the use of these products. In 2009 the US FDA issued a formal warning to consumers to discontinue use of three nasally administered versions of Zicam Cold Remedy. This incident is more troubling since zinc-induced anosmia has been recognized in people since the 1950's and it is a well-recognized nasal toxicant in animals (Dorman, 2010).

Although the safety and efficacy of the proton pump inhibitors are well known, their effects on olfaction are not established. Preclinical assessment of the nasal/olfactory toxicity of a drug is limited. One published French study reports that some people taking esomeprazole (Nexium) developed cacosmia (i.e., reporting a foul or rotten smell when none should be present) (Marie et al., 2005). In general, the mechanisms underlying drug-induced taste and/or smell alterations can be classified into two groups; (a) primary mechanisms resulting from a direct action of the drug; and (b) secondary mechanisms, in which the altered perception is consequent to collateral effects of the drug (Tuccori et al., 2011). To our knowledge the possible mechanism of action for esomepraole-induced cacosmia is not known. It is known that the intracellular pH of the olfactory epithelium is under homeostatic control and may play a role in olfaction (Hu et al., 2007; Turetsky et al., 2009). *The goal of this phase is to evaluate whether prophylactic administration of omeprazole may alter olfaction in dogs.*

Materials and Methods

Animals

Dogs used for this study were housed at K2 (cohort 2). All dogs used in this experiment had been previously trained by K2 staff on AN and C4 and were assessed for olfactory capabilities prior to Prilosec administration (Phase IX). Dogs were trained to detect 8 ounces (227 g) to 3 pounds (1361 g) of AN. Dogs were generally trained to detect 250 g of C4. All dogs were trained to cover on a variety of test odorants including C4 and AN. Dogs were given verbal commands by

their K2 trainers (e.g., “hunt it up”, “go find it”) to begin all searches. Climatic conditions were recorded for each test session. Omeprazole was administered (1 mg/kg/day, oral) 2 hours after the dogs consumed their morning meal. This dosing schedule optimizes the bioavailability of omeprazole. Olfactory testing began one week after the start of omeprazole administration.

Timed trials with AN and C4

We used an approximately 40 m x 40 m field at the K2 facility. The test field had sparse vegetation and was relatively flat. Surrounding trees and other landscape elements helped to define the boundaries of the test area. Trials performed on a given day began from the same start point. Known quantities of AN (25 and 250 g) were placed at a location approximately 25 m from the start. The surface AN samples were placed in a small (2 cm) depression and covered with leaves, sticks, and other vegetative debris. Several additional depressions were prepared similarly (minus AN) and served as false digs. Ten dogs were used for this phase. Two dogs (‘Charlie’ and ‘Cricket’) were used as concurrent controls and were not treated with omeprazole. These dogs had been excluded because they were in heat during our previous experiments. The time required to detect the AN, as well as the number of true positive and false negative odor covers were recorded. Dogs had a minimum of 30 minutes to rest between trials.

We also assessed the ability of dogs to detect AN that was buried in the 40 x 40 m test field. In this experiment, 250 g of AN was buried 8 cm below the surface of the top soil layer. The AN samples were placed in a nylon mesh bag, buried under the soil, and the small disturbance was covered with leaves, sticks, and other vegetative debris. Several additional negative disturbances were prepared similarly (with vinyl mesh minus AN) and served as false digs.

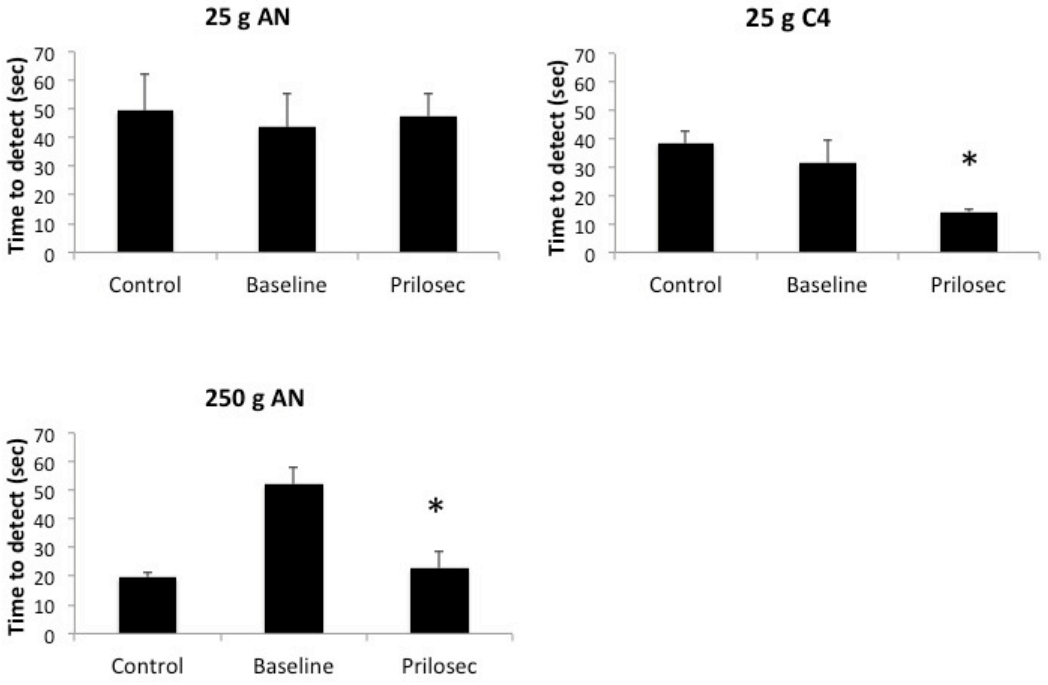
Data analysis

Data collected for both surface and buried AN trials and surface AN trials included: (a) the time required for each dog to detect the odor source; (b) positive and false negative cover rates; and general environmental conditions. All trials were terminated after 180 seconds. In the event that a trial was timed out a value of 180 seconds was used for data analysis. All data collected from the dogs was compared with their baseline data collected during Phase IX. Individual mean values were calculated for each animal and were used in these analyses (n = 2 to 3 trials/dog/agent). Data from the two concurrent control dogs was used for comparative purposes.

Results

Table 27 provides individual summary data for this experiment. Figure 46 shows the overall effect of Prilosec administration on the time to detect either C4 or AN. We found that Prilosec exposure decreased the time to detect 25 g of C4 and 250 g of AN when compared with the animal’s baseline data. With the exception of the 250 g AN trials, values observed in our concurrent control group were in good agreement with the previously obtained baseline data. Dogs given omeprazole were unable to detect 250 g of AN that was buried under 8 cm of soil (data not shown). This result was similar to that seen during our earlier studies (Phase IX). Likewise, omeprazole administration was not associated with a change in false cover rates (data not shown).

Figure 46. Mean (± SEM) time to detect AN and C4 either 1 month prior to omeprazole administration ('Baseline') or after the start of oral dosing at 1 mg/kg/day, for 7 consecutive days ('Prilosec'). Data from 2 concurrent controls ('Control') is also shown. * $p < 0.05$ (ANOVA, vs 'Baseline' data).



Discussion

Our study shows a possible benefit on odorant detection from omeprazole treatment. A benefit could arise from either improved motivation to work (e.g., reduced signs associated with exercise-induced gastric lesions), improved olfactory function (e.g., changes in airflow patterns, alterations in olfactory neuron function), improved cognition or drive – or some combination. Although direct data is not available we suspect that the improvement seen is likely due to general health benefits rather than changes in odorant detection or signaling.

There are several important caveats that must be considered when evaluating our results. First, drug-induced adverse effects occur rarely in the general human population. We anticipate that the same is true for dogs. It remains possible that some dogs may be susceptible to omeprazole-induced adverse olfactory effects that would go unrecognized in a replicated study like ours with a larger sample size. Second, our experiment only evaluated responses after short-term repeated administration of omeprazole at a therapeutically relevant dose. It is not uncommon for certain adverse effects to emerge after chronic drug administration or in studies that use higher exposure doses. Follow-up studies are indicated as well to confirm the beneficial effects that we have seen in this pilot study.

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Table 1. Dog demographic data.

Name	ID #	Sex[a]	Color[b]	Whelp date	Arrival Date	Spay Date	Location[c]
Ace	426	M	B	1/21/10	1/18/11	N/A	NCSU
Annie	436	F	Y	11/25/09	1/18/11	N/A	NCSU
Baxter	367	M	B	11/29/08	11/16/10	N/A	NCSU
Bullet	715	M	Y	6/21/08	4/18/11	N/A	NCSU
Dakota	349	F	B	10/18/09	11/11/10	N/A	NCSU
Honey	226	F	Y	11/21/09	10/14/10	N/A	NCSU
Hunter	415	M	B	1/5/10	1/14/11	N/A	NCSU
Jimmy	532	SF	B	5/9/09	1/31/11	4/22/11	NCSU
Macks	549	M	B	10/9/09	2/22/11	N/A	NCSU
Mercy	480	SF	B	5/22/09	1/15/11	4/14/11	NCSU
Piper	581	F	Y	2/17/10	2/20/11	N/A	NCSU
Reno	234	M	Y	8/22/09	10/14/10	N/A	NCSU
Rip	416	M	B	1/15/10	1/14/11	N/A	NCSU
Ruby	311	SF	Y	12/15/09	11/7/10	7/8/11	NCSU
Valentine	506	F	B	2/6/10	2/3/11	N/A	NCSU
Wizard	235	M	B	11/29/08	10/14/10	N/A	NCSU
Allie	90	SF	B	8/28/08	2/14/10	11/23/09	K2
Annie	163	SF	B	2/1/10	7/1/10	7/15/11	K2
Brutus	851	M	C	8/12/10	9/15/11	N/A	K2
Charlie	738	F	B	4/15/10	6/17/11	N/A	K2
Cricket	361	F	B	1/24/10	11/12/10	N/A	K2
Hannah	452	SF	B	2/1/09	1/14/11	7/12/11	K2
Harley	479	M	B	2/2/09	1/18/11	N/A	K2
Heidi	437	SF	Y	5/26/07	2/7/11	7/15/11	K2
Ike	682	M	B	7/27/07	4/7/11	N/A	K2
Kody	649	SF	Y	3/15/08	4/18/11	8/2/11	K2
Salty	423	F	B	4/17/10	1/18/11	N/A	K2
Twiggy	850	F	B	4/7/10	9/15/11	N/A	K2

[a]F = female, SF = spayed female, M = male
[b]B = black, C = chocolate, Y = yellow
[c]Dogs held at NCSU comprise cohort 1. Dogs held at K2 comprise cohort 2.

Table 2. Prior training experience at K2

Name	IDD Status	IDD Reason	Days of training	Behavior issues	Odors	Age on Nov. 1, 2011	USMC ERT
Cohort 1							
Ace	Failed	Imprint	33	Lacked confidence in directional control training	C4,DC,TNT, AN,SC,PC	1y, 10 mo	1/31/11, 8/10/11
Annie	None	Minimal training	None	Lack of confidence in training, no force fetch	None	1y, 11 mo	1/24/11, and 2nd
Baxter	None	Minimal training	None	None	None	2y, 11 mo	None
Bullet	None	Minimal training	None	Failed directional control	None	3y, 4 mo	4/24/11
Dakota	None	Minimal training	None	Failed directional control	None	2y, 0 mo	None
Honey	None	Minimal training	None	None	None	1y, 11 mo	None
Hunter	None	Failed directional control	5	Worrisome, sit is weak, undue pressure, poor attitude	None	1y, 10 mo	1/24/11
Jimmy	Failed	2X cert failure	86	Nervous around gunfire	AN,C4,TNT, PC,SC,DC	2y, 6 mo	No date
Macks	Failed	Heartworm, currently negative	52	None	AN,C4,TNT, PC,SC,DC	2y, 1 mo	3/7/11, 4/18/11
Mercy	Failed	2X cert failure	91	Poor hunting, 'soft'	AN,DC,TNT, PC,SC,C4	2y, 5 mo	None
Piper	None	Minimal directional control training	None	None	None	1y, 8 mo	2/21/11
Reno	None	Minimal directional control training	None	Failed directional control	None	2y, 2mo	11/1/10
Rip	Failed	Lack hunting skills and directional control training	33	Poor attitude in training	C4,TNT,DC, AN,SC,PC	1y, 9 mo	1/21/11
Ruby	Failed	2X cert failure	85	None	AN,C4,DC, TNT,SC,PC	1y, 10 mo	6/6/11
Valentine	None	Minimal retriever directional control training	None	Lacks confidence in new environments	None	1y, 9 mo	2/14/11
Wizard	None	Minimal basic retriever directional control training	None	None	None	2y, 11 mo	11/8/10

Odors: AN (ammonium nitrate), PC (potassium chlorate), SC (sodium chlorate), DC (detonating cord

Table 2. Prior training experience at K2 (Continued)

Name	IDD Status	IDD Reason	Days of training	Behavior issues	Odors	Age on Nov. 1, 2011	USMC ERT
Cohort 2							
Allie	Failed	Cruciate surgery 10/18/10 just before cert.	N/A	Hyperactive	No records	3y, 2 mo	None
Annie	Failed	Imprint	69	Soft dog, retriever skills, might bolt	C4,DC,TNT, AN,SC,PC	1y, 9 mo	None
Brutus	Failed	Transvert and inc. sacral fusion	26	Insecure in some new environments, good on odors	AN partial	1y, 3 mo	9/20/11
Charlie	Failed	Heartworm	18	None	None	1y, 6 mo	6/21/11
Cricket	Failed	Environmental acclimation, nervous	4 IDD, 90 total	Worrisome, nervous, fearful, licks and salivates in truck	None	1y, 9 mo	None
Hannah	Failed	Imprint on odor wall	27	None	AN, did not complete	2y, 9 mo	6/6/11
Harley	Failed	Gastrointestinal, couldn't hold wt., no problem at VTH	21	None	None	2y, 9 mo	1/24/11
Heidi	Failed	Age, ready for cert.	70	None	TNT,C4,AN, DC,PC,SC	4y, 5 mo	Blank forms
Ike	Failed	2X cert failure	84	Lazy	AN, PC, SC, DC, TNT, C4	4y, 3 mo	4/14/11
Kody	Failed	Medical, early DJD	54	Green for retriever skills, clingy, 'too obedient'	C4,TNT,DC, AN,PC,SC	3y, 7 mo	4/24/11
Salty	Failed	Medical, inability to flex toe on one hind paw	30	None	C4,TNT,AN, PC,SC,DC	1y, 6 mo	No date & 8/10/11
Twiggy	Failed	Trans vertebrae spondylosis, fair hips	26	None	C4,TNT,AN, PC,SC,DC	1y, 7 mo	9/20/11

Odors: AN (ammonium nitrate), PC (potassium chlorate), SC (sodium chlorate), DC (detonating cord)

Table 3. ERT ordinal scores

1a Stranger Exam - Initial Contact
8a Stranger Exam - Initial Contact - repeat
1 Dog does not make contact with stranger.
2 Dog makes contact after stranger crouches down and speaks to dog.
3 Dog makes contact when handler is next to stranger.
4 Dog makes contact as soon as handler moves toward stranger.
5 Dog approaches stranger immediately and independently.

1b Stranger Exam
8b Stranger Exam - repeat
1 Dog actively attempts to escape or growls and threatens.
2 Dog withdraws or shrinks away from person, nervousness. Note if dog is flank shy.
3 Dog accepts exam, indifferent to stranger.
4 Dog accepts exam, attentive to stranger.
5 Dog accepts exam, actively seeks play with stranger, excited.

2 Stairs and Surface - Up
1 Dog refuses to ascend stairs, cannot be motivated to proceed up stairs.
2 Dog requires active and continuous motivation to proceed up stairs.
3 Dog, with initial handler encouragement, moves up stairs, tentative (low, "slinky" posture).
4 Dog hesitates before or upon stepping on stair, and then moves easily.
5 Dog moves onto and up stairs without hesitation.

3a Visual Startle - Bag Drop
1 Dog is fearful, bolts to end of leash facing away or turns away from object.
2 Dog is startled, steps backward, pronounced movement/retreat, faces object.
3 Dog stops and crouches or flinches, may step back, remains facing object.
4 Dog stops briefly, transient reaction, recovers quickly.
5 Dog shows no fear reaction to being startled.

3b Recovery from Visual Startle - Approach Bag
1 Dog refuses to approach object despite handler motivation.
2 Dog requires handler motivation to approach object.
3 Dog approaches hesitantly (start/stop avoidance), angles toward object on curving path.
4 Dog approaches cautiously but directly.
5 Dog approaches immediately without hesitation.

4a Acoustic Startle - Grate 1 in front of dog;
4c Acoustic Startle - Grate 2 behind dog
1 Dog is fearful, bolts to end of leash facing away or turns away from sound.
2 Dog is startled, steps backward, pronounced movement/retreat, faces sound.
3 Dog stops and crouches or flinches, may step back, remains facing sound.
4 Dog stops briefly, transient reaction, recovers quickly.
5 Dog shows no fear reaction to being startled.

4b Recovery from Acoustic Startle - Approach Grate 1
1 Dog refuses to approach grate despite handler motivation.
2 Dog requires handler motivation to approach grate.
3 Dog approaches hesitantly (start/stop avoidance), angles toward grate on curving path.
4 Dog approaches grate cautiously but directly.
5 Dog approaches grate immediately without hesitation.

5a Novel Object - Remote Control Vehicle

1 Dog retreats behind handler immediately and remains.
2 Dog retreats behind handler after initial approach.
3 Dog retreats (steps back), but not further than handler's side, remains facing object.
4 Dog steps back (mild retreat) then approaches, shows intermittent displacement behaviors.
5 Dog shows no fear reaction to car movement, no withdrawal.

5b Approach Remote Control Vehicle

1 Dog refuses to approach object despite handler motivation.
2 Dog requires handler motivation to approach object.
3 Dog approaches hesitantly (start/stop avoidance), angles toward object on curving path.
4 Dog approaches cautiously but directly.
5 Dog approaches immediately without hesitation.

Table 3. ERT ordinal scores (Continued)

6a Unusual Stranger Test - Fear
1 Dog escapes behind handler before stranger is 1/2 of distance.
2 Dog escapes behind handler after stranger is 1/2 of distance.
3 Dog retreats (step back), but never further back than handler's side.
4 Dog shows intermittent displacement behaviors (lip licking, yawn, sniff, looks away).
5 Dog shows little or no reaction to stranger, no withdrawal.

6b Unusual Stranger - Aggression
1 Dog attacks before stranger is 1/2 distance to dog.
2 Dog shows repeated aggression with attack after stranger is 1/2 the distance.
3 Dog shows repeated aggression and threats, but no attack.
4 Dog shows mild, intermittent aggression.
5 Dog shows no aggression.

6c Unusual Stranger Recovery - Dog Approach
1 Dog does not make contact with stranger.
2 Dog makes contact after stranger crouches down and speaks to dog.
3 Dog makes contact when handler is next to stranger.
4 Dog makes contact as soon as handler moves toward stranger.
5 Dog approaches stranger immediately and independently.

7 Stairs and Surface - Down
1 Dog refuses to descend stairs, cannot be motivated to proceed down stairs.
2 Dog requires active and continuous motivation to proceed down stairs.
3 Dog, with initial handler encouragement, moves down stairs, tentative (low posture)
4 Dog hesitates before or upon stepping on stair, and then moves easily.
5 Dog moves onto and down stairs without hesitation.

9 Stranger with Umbrella Startle
1 Dog is fearful, bolts to end of leash facing away or turns away from object.
2 Dog is startled, steps backward, pronounced movement/retreat, faces object.
3 Dog stops and crouches or flinches, may step back, remains facing object.
4 Dog stops briefly, transient reaction, may crouch or flinch, recovers quickly.
5 Dog shows no fear reaction to being startled.

10a Gunfire Test 1 - 100 ft. away
10b Gunfire Test 2 - 75 ft. away
10c Gunfire Test 3 - 50 ft. away
1 Dog is fearful, bolts to end of leash, marked escape attempt.
2 Dog fearful, some effort to escape / retreat, avoidance.
3 Dog is mildly fearful, may show anxiety behaviors, crouching, no recovery.
4 Dog orients toward sound, stops briefly, transient reaction, recovers quickly.
5 Dog shows no fear reaction to being startled.

10d Gunfire Test - overall /global
1 Dog flees, escapes during walk.
2 Dog shows increase in behavior change and escape behaviors after shots.
3 Dog shows nervousness and cannot continue activity.
4 Dog orients toward sound, startles but normal behavior always resumes.
5 Dog is not affected, activity remains uninterrupted.

Table 4. Summary of the ERT results for cohort 1.

Name	Sex	NCSU ERT	NCSU ERT Anxiety	NCSU USMC ERT[a]
Ace	M	114	96	55
Annie	F	86	67	38
Baxter	M	98	81	44
Bullet	M	114	91	57
Dakota	F	109	90	54
Honey	F	76	63	34
Hunter	M	109	89	56
Jimmy	SF	108	88	53
Macks	M	99	84	50
Mercy	SF	111	90	56
Piper	F	67	54	26
Reno	M	106	87	49
Rip	M	105	87	49
Ruby	SF	109	92	53
Valentine	F	91	72	42
Wizard	M	102	79	51

[a]Performed by NCSU scientists

Table 5. Summary of the ERT results for cohort 2.

Name	Sex	NCSU ERT	USMC ERT[a]
Allie	SF	101	53
Annie	SF	90	50
Brutus	M	61	39
Charlie	F	77	42
Cricket	F	38	22
Hannah	SF	80	44
Harley	M	104	56
Heidi	SF	93	51
Ike	M	93	51
Kody	SF	103	56
Salty	F	90	52
Twiggy	F	97	54

[a]Performed by NCSU scientists

Table 6. Individual test results for the ERT performed on cohort 1.

Name	Stairs1 up	Grate footing 1	Ramp1	Crowd 1	Crowd 2	Ramp 2	Grate footing 2	Stairs 2 down	Stranger exam approach	Stranger exam	Visual startle bag	Visual startle approach
Ace	4	5	5	4	5	5	5	5	5	2	5	5
Annie	3	4	5	4	4	5	5	5	5	4	1	2
Baxter	2	4	5	3	3	5	5	5	5	3	3	3
Bullet	4	5	5	5	5	5	5	5	5	4	5	5
Dakota	5	5	5	4	5	4	5	5	5	3	5	5
Honey	2	2	5	2	2	5	4	5	5	2	2	3
Hunter	5	5	5	4	5	5	5	5	5	3	5	5
Jimmy	4	5	5	3	3	5	5	5	5	5	5	5
Macks	4	5	5	4	2	5	5	4	5	2	4	4
Mercy	4	4	5	5	5	4	4	4	5	2	5	5
Piper	4	5	5	2	2	5	5	5	4	2	1	2
Reno	3	5	4	4	4	4	5	5	5	3	4	5
Rip	5	5	5	3	4	5	5	5	5	3	5	5
Ruby	5	5	5	3	3	5	5	5	5	3	5	5
Valentine	2	4	5	4	4	5	4	5	3	3	4	5
Wizard	2	3	5	4	4	2	4	5	5	5	5	5

Table 6. Individual test results for the ERT performed on cohort 1 (Continued).

Name	Acoustic startle front	Acoustic startle approach	Acoustic startle behind	Unusual stranger fear	Unusual stranger aggression	Unusual stranger approach	Stranger exam 2 contact	Stranger exam 2	Umbrella	Remote vehicle	Remote vehicle approach	Gunfire low dB	Gunfire high dB
Ace	5	5	5	5	5	5	5	2	4	4	4	5	5
Annie	3	3	3	3	4	2	5	3	2	2	2	3	4
Baxter	4	4	3	4	5	4	5	3	5	4	4	3	4
Bullet	5	5	4	5	5	5	5	4	3	4	3	4	4
Dakota	4	4	5	5	5	5	5	2	5	3	2	4	4
Honey	3	3	3	3	5	1	5	2	1	3	2	3	3
Hunter	5	5	4	5	5	5	5	3	2	3	2	4	4
Jimmy	4	4	3	5	5	5	4	4	4	4	3	4	4
Macks	4	5	4	3	4	2	5	3	3	4	3	5	5
Mercy	5	5	4	5	5	5	5	4	4	3	4	5	5
Piper	2	1	2	2	5	1	1	2	1	1	1	3	3
Reno	4	5	5	5	5	1	5	3	5	4	4	4	5
Rip	3	3	4	5	5	2	5	3	4	4	2	5	5
Ruby	5	5	4	4	5	4	5	3	5	4	3	4	4
Valentine	2	1	2	5	5	2	5	3	4	3	3	4	4
Wizard	4	3	4	4	5	5	5	5	4	3	3	4	4

Table 7. Individual test results for the ERT performed on cohort 2.

Name	Crowd 1	Crowd 2	Stranger exam approach	Stranger exam	Up stairs	Visual startle bag	Visual startle approach	Acoustic startle front	Acoustic startle approach	Acoustic startle behind	Car	Car approach
Allie	3	4	5	4	5	5	5	4	5	4	3	4
Annie	4	3	5	4	5	4	5	4	4	4	4	4
Brutus	2	3	2	3	5	4	4	2	3	2	1	2
Charlie	3	4	5	2	5	4	4	3	4	2	1	1
Cricket	4	3	4	3	5	1	2	1	1		1	1
Hannah	4	3	4	4	4	4	4	3	2	3	2	3
Harley	3	4	5	4	5	5	5	5	5	5	3	2
Heidi	4	3	4	3	5	4	5	5	5	4	2	3
Ike	4	3	3	4	5	4	4	4	5	4	3	3
Kody	4	4	3	4	5	5	5	5	5	5	4	5
Salty	4	5	5	4	4	5	5	3	4	2	2	2
Twiggy	4	3	5	3	5	5	5	4	5	3	4	4

Table 7. Individual test results for the ERT performed on cohort 2 (Continued).

Name	Unusual stranger fear	Unusual stranger aggression	Unusual stranger approach	Down stairs	Stranger exam 2 contact	Stranger exam 2	Umbrella	Gunfire 100'	Gunfire 75'	Gunfire 50'	Gunfire overall
Allie	4	5	4	5	4	4	4	5	5	5	5
Annie	4	5	4	5	5	4	5	2	2	2	2
Brutus	4	5	3	5	3	3	3	1			1
Charlie	4	5	1	4	2	2	1	5	5	5	5
Cricket	1	5		5	1						
Hannah	4	5	1	3	5	4	1	4	4	5	4
Harley	5	5	5	5	4	5	4	5	5	5	5
Heidi	4	5	3	5	4	3	5	4	4	5	4
Ike	5	5	2	5	5	4	5	4	4	4	4
Kody	4	5	5	5	4	3	4	4	5	5	5
Salty	4	5	5	5	5	2	2	4	4	5	4
Twiggy	5	5	5	5	4	3	4	4	4	4	4

Table 8. Cortisol levels (µg/dL) for ERT (cohort 1)

		Plasma		**Saliva**	
Dog	Sex	Baseline	Post-ERT	Baseline	Post-ERT
Ace	M	1.42	1.63	0.253	0.186
Annie	F	1.05	2.21	0.182	0.256
Baxter	M	1.97	4.55	0.189	0.228
Bullet	M	<1.00	2.50	0.165	0.115
Dakota	F	1.27	2.22	0.110	0.195
Honey	F	2.22	4.15	0.148	0.166
Hunter	M	<1.00	1.46	0.228	0.373
Jimmy	SF	1.68	3.70	0.135	0.245
Macks	M	<1.00	1.15	0.090	0.192
Mercy	SF	<1.00	1.83	0.231	0.103
Piper	F	1.05	5.41	0.123	0.240
Reno	M	<1.00	4.02	0.195	0.158
Rip	M	1.40	2.19	0.147	0.270
Ruby	SF	1.39	1.83	0.132	0.139
Valentine	F	1.31	5.77	0.121	0.222
Wizard	M	1.28	2.37	0.092	0.156

Baseline samples were collected one week before the ERT. Note: a value of 0.99 was assigned for samples below the plasma cortisol assay detection limit (<1).

Table 9. Mean (± SEM) total distance traveled for male and female dogs during the five-day open field test. The daily order of the stimuli were no sound (day 1), thunderstorm (day 2), no sound (day 3), recorded gunfire (day 4), and no sound (day 5). On days 2 and 4, the sound stimuli were presented during the middle 3 minutes of the 9 minute test session.

	Total Distance (m) Traveled (mean ±SEM)	
Session	Female	Male
Control 1	96.7 ± 31.9	103.8 ± 41.8
Control 2	28.6 ± 17.0	71.3 ± 45.7
Control 3	19.5 ± 10.6	76.5 ± 51.8
Thunderstorm	50.4 ± 25.2	87.9 ± 41.8
Gunfire	24.7 ± 17.3	60.7 ± 35.0

Table 10. Ethovision analysis of the open-field test

Name	OFT date	Total Distance moved (m)	Mean Velocity (m/min)	Door Zone Duration (min)	Hidden Zone Duration (min)	Front Wall Zone Duration (min)	Center-point / Not Moving Duration (min)
Ace	03/05/2012	379.091	42.113	6.035	0.045	6.272	0.825
Annie	03/05/2012	132.153	14.681	6.6	0.028	6.828	5.568
Baxter	03/12/2012	7.356	0.817	8.458	-	8.393	8.617
Bullet	03/05/2012	54.949	6.104	7.538	-	3.707	6.505
Dakota	03/12/2012	27.108	3.011	7.745	0.038	7.175	7.943
Honey	03/05/2012	16.934	1.881	8.485	-	8.41	8.178
Hunter	03/12/2012	79.267	13.102	3.747	0.267	3.567	2.578
Jimmy	03/05/2012	14.641	1.626	8.825	-	8.925	8.388
Macks	03/12/2012	97.324	10.812	7.568	0.037	7.345	5.882
Mercy	03/05/2012	286.87	31.869	5.007	-	5.19	0.977
Piper	03/12/2012	84.32	9.367	5.813	-	5.227	5.308
Reno	03/12/2012	22.309	2.478	5.558	-	4.582	7.8
Rip	03/12/2012	131.668	14.627	6.677	0.247	6.212	4.113
Ruby	03/12/2012	79.865	8.872	6.367	-	6.18	6.167
Valentine	03/05/2012	131.87	14.65	5.822	-	5.943	4.252
Wizard	03/05/2012	58.413	6.489	7.63	0.033	2.803	7.352

Table 10. Ethovision analysis of the open-field test (Continued)

Name	OFT date	Total Distance moved (m)	Mean Velocity (m/min)	Door Zone Duration (min)	Hidden Zone Duration (min)	Front Wall Zone Duration (min)	Center-point / Not Moving Duration (min)
Day 2							
Ace	03/06/2012	371.617	41.283	6.388	0.028	7.335	0.583
Annie	03/06/2012	31.646	3.516	8.553	-	8.575	8.112
Baxter	03/13/2012	13.131	1.459	8.358	-	8.077	8.398
Bullet	03/06/2012	57.092	6.342	7.662	-	2.505	6.755
Dakota	03/13/2012	9.876	1.097	8.695	-	8.155	8.652
Honey	03/06/2012	4.253	0.472	9.003	-	9.003	8.957
Hunter	03/13/2012	69.395	7.709	7.058	0.565	6.332	5.98
Jimmy	03/06/2012	15.551	1.728	8.847	-	8.968	8.448
Macks	03/13/2012	24.807	2.756	8.6	-	8.65	8.128
Mercy	03/06/2012	220.736	24.522	6.603	-	7.303	2.588
Piper	03/13/2012	46.671	5.185	7.628	-	3.745	7.165
Reno	03/13/2012	15.104	1.678	8.423	-	8.632	8.308
Rip	03/13/2012	97.535	10.835	7.818	0.127	7.705	5.447
Ruby	03/13/2012	15.609	1.734	8.802	-	8.89	8.38
Valentine	03/06/2012	59.041	6.559	8.33	-	8.472	6.947
Wizard	03/06/2012	54.181	6.019	8.042	0.042	8.457	7.265

Table 10. Ethovision analysis of the open-field test (Continued)

Name	OFT date	Total Distance moved (m)	Mean Velocity (m/min)	Door Zone Duration (min)	Hidden Zone Duration (min)	Front Wall Zone Duration (min)	Center-point / Not Moving Duration (min)
Day 3							
Ace	03/07/2012	386.657	42.954	7.035	-	8.233	0.628
Annie	03/07/2012	8.648	0.961	8.995	-	8.907	8.642
Baxter	03/14/2012	4.591	0.51	0.178	-	0.238	8.805
Bullet	03/07/2012	8.501	0.944	9.003	-	8.988	8.673
Dakota	03/14/2012	1.663	0.185	9.003	-	9.003	8.977
Honey	03/07/2012	2.548	0.283	9.003	-	9.003	8.982
Hunter	03/14/2012	55.217	6.134	7.417	0.325	7.06	6.578
Jimmy	03/07/2012	12.694	1.41	8.9	-	8.873	8.557
Macks	03/14/2012	5.948	0.661	8.952	-	8.937	8.81
Mercy	03/07/2012	145.567	16.171	8.34	-	8.313	4.147
Piper	03/14/2012	24.749	2.749	7.98	-	8.727	8.035
Reno	03/14/2012	14.454	1.606	7.907	-	7.937	8.272
Rip	03/14/2012	35.406	3.933	8.618	0.025	8.598	7.745
Ruby	03/14/2012	22.63	2.514	8.565	-	8.532	8.087
Valentine	03/07/2012	10.605	1.178	8.887	-	8.933	8.63
Wizard	03/07/2012	59.498	6.61	6.525	0.055	5.657	7.35

Table 10. Ethovision analysis of the open-field test (Continued)

Name	OFT date	Total Distance moved (m)	Mean Velocity (m/min)	Door Zone Duration (min)	Hidden Zone Duration (min)	Front Wall Zone Duration (min)	Center-point / Not Moving Duration (min)
Day 4							
Ace	03/08/2012	295.007	32.773	6.832	0.028	7.918	1.6
Annie	03/08/2012	4.761	0.529	9.003	-	8.992	8.922
Baxter	03/15/2012	7.054	0.784	5.657	-	5.705	8.817
Bullet	03/08/2012	11.488	1.276	8.95	-	7.11	8.583
Dakota	03/15/2012	1.149	0.128	9.003	-	9.003	8.978
Honey	03/08/2012	5.293	0.588	9.003	-	9.003	8.875
Hunter	03/15/2012	55.107	6.122	7.275	0.247	7.453	6.728
Jimmy	03/08/2012	2.544	0.283	9.003	-	9.003	8.918
Macks	03/15/2012	4.808	0.534	9.003	-	8.982	8.835
Mercy	03/08/2012	143.31	15.92	7.517	-	8.222	4.528
Piper	03/15/2012	9.934	1.104	8.938	-	9.003	8.575
Reno	03/15/2012	14.18	1.575	6.943	-	3.733	8.27
Rip	03/15/2012	12.506	1.389	8.743	-	8.715	8.522
Ruby	03/15/2012	28.944	3.215	8.675	-	8.728	7.942
Valentine	03/08/2012	1.396	0.155	9.003	-	9.003	9.002
Wizard	03/08/2012	85.541	9.503	8.028	0.042	8.258	6.482

Table 10. Ethovision analysis of the open-field test (Continued)

Name	OFT date	Total Distance moved (m)	Mean Velocity (m/min)	Door Zone Duration (min)	Hidden Zone Duration (min)	Front Wall Zone Duration (min)	Center-point / Not Moving Duration (min)
Day 5							
Ace	03/09/2012	434.849	48.308	6.76	-	8.673	0.513
Annie	03/09/2012	3.072	0.341	8.942	-	8.93	8.897
Baxter	03/16/2012	2.671	0.297	8.91	-	0.385	8.89
Bullet	03/09/2012	11.467	1.274	8.97	-	8.592	8.553
Dakota	03/16/2012	1.242	0.138	9.003	-	9.003	8.977
Honey	03/09/2012	1.642	0.182	9.003	-	9.003	8.957
Hunter	03/16/2012	35.749	3.971	3.002	4.960	2.932	7.295
Jimmy	03/09/2012	4.243	0.471	9.003	-	7.743	8.785
Macks	03/16/2012	1.137	0.126	9.003	-	9.003	8.972
Mercy	03/09/2012	86.617	9.622	8.508	-	8.473	6.642
Piper	03/16/2012	22.474	2.497	2.715	-	2.69	7.947
Reno	03/16/2012	12.656	1.406	7.153	-	6.76	8.345
Rip	03/16/2012	46.312	5.145	8.73	0.025	8.68	7.317
Ruby	03/16/2012	35.06	3.895	7.943	-	7.392	7.447
Valentine	03/09/2012	1.657	0.184	9.003	-	9.003	8.972
Wizard	03/09/2012	66.764	7.417	5.497	0.213	4.987	6.82

Table 11. Physiologic responses of dogs during the open-field test

Dog Name	Heart rate Pre	Heart rate Post	Δ HR Pre-Post	Temp (°F) Pre	Temp (°F) Post	Δ Temp Pre-Post
Ace	97	121	24	101.8	103.2	1.4
Annie	113	99	-14	102.7	102.9	0.2
Baxter	104	102	-2	104.1	102.4	-1.7
Bullet	98	101	3	102.2	101.5	-0.7
Dakota	111	98	-13	103	101.9	-1.1
Honey	118	116	-2	103	102.7	-0.3
Hunter	120	115	-5	101.9	103.7	1.8
Jimmy	85	92	7	102.7	102.9	0.2
Macks	90	88	-2	100.9	101.3	0.4
Mercy	104	130	26	103.3	102.8	-0.5
Piper	106	102	-4	101.9	102.5	0.6
Reno	106	101	-5	103.3	103.2	-0.1
Rip	99	92	-7	101.7	101.9	0.2
Ruby	103	111	8	101.4	102.7	1.3
Valentine	105	103	-2	102.7	102.7	0
Wizard	95	112	17	103	102.6	-0.4

Table 11. Physiologic responses of dogs during the open-field test (Continued)

Dog Name	Heart rate Pre	Heart rate Post	Δ HR Pre-Post	Temp (°F) Pre	Temp (°F) Post	Δ Temp Pre-Post
Day 2						
Ace	95	109	14	102.1	102.3	0.2
Annie	102	101	-1	103.1	102.7	-0.4
Baxter	92	79	-13	101.5	101.3	-0.2
Bullet	108	99	-9	101.5	101	-0.5
Dakota	119	103	-16	102.7	101.6	-1.1
Honey	108	109	1	102.5	103.2	0.7
Hunter	118	111	-7	101.8	102.1	0.3
Jimmy	109	114	5	102.2	102.2	0
Macks	89	75	-14	101.2	101	-0.2
Mercy	105	104	-1	103.3	103.2	-0.1
Piper	109	103	-6	101.1	101.7	0.6
Reno	100	99	-1	102.8	102.7	-0.1
Rip	97	100	3	101.7	101.6	-0.1
Ruby	101	102	1	100.7	101.1	0.4
Valentine	102	94	-8	102.2	101.7	-0.5
Wizard	88	102	14	102.6	101.5	-1.1

Table 11. Physiologic responses of dogs during the open-field test (Continued)

Dog Name	Heart rate Pre	Heart rate Post	Δ HR Pre-Post	Temp (°F) Pre	Temp (°F) Post	Δ Temp Pre-Post
Day 3						
Ace	114	116	2	102.4	102	-0.4
Annie	100	100	0	102.8	102.3	-0.5
Baxter	99	91	-8	103.5	102.5	-1
Bullet	103	101	-2	102.2	101.5	-0.7
Dakota	93	88	-5	100.2	100.3	0.1
Honey	107	88	-19	101.3	101.7	0.4
Hunter	108	90	-18	101.7	101.9	0.2
Jimmy	106	101	-5	102.1	102.6	0.5
Macks	82	76	-6	101.1	101	-0.1
Mercy	125	131	6	102.4	103	0.6
Piper	89	86	-3	101.3	101.8	0.5
Reno	104	108	4	103.7	102.9	-0.8
Rip	86	81	-5	101.6	101.6	0
Ruby	94	82	-12	100.3	100.2	-0.1
Valentine	112	94	-18	102.5	102	-0.5
Wizard	128	111	-17	102.2	102	-0.2

Table 11. Physiologic responses of dogs during the open-field test (Continued)

Dog Name	Heart rate Pre	Heart rate Post	Δ HR Pre-Post	Temp (°F) Pre	Temp (°F) Post	Δ Temp Pre-Post
Day 4						
Ace	100	110	10	101.5	102	0.5
Annie	112	108	-4	101.8	102.3	0.5
Baxter	101	84	-17	103.4	102.7	-0.7
Bullet	105	99	-6	102.1	101.6	-0.5
Dakota	106	108	2	102.7	101.6	-1.1
Honey	121	109	-12	102.3	102.5	0.2
Hunter	118	95	-23	101.9	102.1	0.2
Jimmy	94	105	11	101.4	101.6	0.2
Macks	85	80	-5	101.4	101.6	0.2
Mercy	125	118	-7	102	102.5	0.5
Piper	102	95	-7	101.6	101.9	0.3
Reno	108	104	-4	101.8	102	0.2
Rip	100	89	-11	101.1	101.4	0.3
Ruby	95	84	-11	100.5	100.9	0.4
Valentine	98	106	8	102.9	102.6	-0.3
Wizard	108	106	-2	101.7	101.5	-0.2

Table 11. Physiologic responses of dogs during the open-field test (Continued)

Dog Name	Heart rate Pre	Heart rate Post	Δ HR Pre-Post	Temp (°F) Pre	Temp (°F) Post	Δ Temp Pre-Post
Day 5						
Ace	114	126	12	103.9	102.9	-1
Annie	100	91	-9	103.2	102.7	-0.5
Baxter	96	79	-17	102.2	101.5	-0.7
Bullet	105	98	-7	102.3	101.3	-1
Dakota	112	105	-7	101.3	101.4	0.1
Honey	111	99	-12	102.2	102.5	0.3
Hunter	113	100	-13	102.1	101.9	-0.2
Jimmy	112	100	-12	100.8	101.4	0.6
Macks	100	85	-15	100.2	100.8	0.6
Mercy	122	115	-7	102.5	102.6	0.1
Piper	112	89	-23	101.4	101.9	0.5
Reno	105	119	14	103.3	102.9	-0.4
Rip	111	104	-7	101.7	101.7	0
Ruby	88	80	-8	100.5	100.8	0.3
Valentine	108	98	-10	102.4	101.7	-0.7
Wizard	122	110	-12	101.4	101.5	0.1

Table 12. Salivary cortisol levels (µg/dL) for open-field test at NCSU

Dog	Sex	Day 1	Day 2 (TS)	Day 3	Day 4 (GF)	Day 5
Ace	M	0.294	0.362	0.397	0.435	0.551
Annie	F	0.193	0.207	0.180	0.192	0.222
Baxter	M	0.271	0.345	0.237	0.218	0.218
Bullet	M	0.246	0.332	0.234	0.207	0.376
Dakota	F	0.186	0.226	0.223	0.324	0.276
Honey	F	0.311	0.309	0.291	0.226	0.236
Hunter	M	0.523	0.260	0.229	0.254	0.218
Jimmy	SF	0.247	0.187	0.222	0.203	0.315
Macks	M	0.366	0.344	0.370	0.230	0.293
Mercy	SF	0.225	0.211	0.131	0.193	0.244
Piper	F	0.183	0.179	0.109	0.230	0.098
Reno	M	0.244	0.100	0.243	0.257	0.240
Rip	M	0.298	0.579	0.195	0.251	0.277
Ruby	SF	0.305	0.195	0.128	0.177	0.137
Valentine	F	0.199	0.321	0.205	0.289	0.363
Wizard	M	0.095	0.139	0.155	0.160	0.161

Saliva was collected following each 9 minute open-field session.
Day 2 (TS) – Thunderstorm sounds during middle 3 minutes
Day 4 (GF) – Gunfire battle sounds during middle 3 minutes

Table 13. Mean (± SEM) anxiety scores for male and female dogs during the five-day open field test. The daily order of the stimuli were no sound (day 1), thunderstorm (day 2), no sound (day 3), recorded gunfire (day 4), and no sound (day 5). On days 2 and 4, the sound stimuli was presented during the middle 3 minutes of the 9 minute test session.

		Mean Global Scores (mean ±SEM)	
Session	Epoch	Female	Male
Control 1	Before	3.69 ± 0.48	3.56 ± 0.89
	During	3.31 ± 1.04	3.47 ± 1.14
	After	2.78 ± 1.14	3.11 ± 1.31
Control 2	Before	2.72 ± 1.24	3.22 ± 1.22
	During	2.59 ± 1.25	2.91 ± 1.39
	After	2.34 ± 1.20	2.72 ± 1.28
Control 3	Before	2.28 ± 1.44	3.06 ± 1.19
	During	1.97 ± 1.19	2.81 ± 1.16
	After	1.88 ± 1.13	2.63 ± 1.23
Thunderstorm	Before	2.81 ± 1.12	3.38 ± 0.94
	During	4.25 ± 0.69	4.03 ± 0.63
	After	2.91 ± 0.86	3.31 ± 0.83
Gunfire	Before	1.97 ± 1.12	2.78 ± 1.21
	During	3.44 ± 1.27	4.25 ± 0.74
	After	2.22 ± 1.34	3.06 ± 1.19

Table 14. Behavioral (anxiety) responses of dogs during the open-field test

Dog Name	Mean Global Score 1 Pre	Mean Global Score 2 During	Mean Global Score 3 Post	Mean Sessions 1-3	% Change Anxiety (During/pre)	% Change Anxiety (Post/pre)
Day 1						
Ace	4.25	4.25	4.25	4.25	100.00	100.00
Annie	4	2.5	1.5	2.67	62.50	37.50
Baxter	2.5	1.5	1	1.67	60.00	40.00
Bullet	3.25	3.25	3	3.17	100.00	92.31
Dakota	3.5	3.5	2.5	3.17	100.00	71.43
Honey	3.5	3.5	3	3.33	100.00	85.71
Hunter	4.25	4.75			111.76	
Jimmy	3	1.5	1.25	1.92	50.00	41.67
Macks	2.25	2.25	1.75	2.08	100.00	77.78
Mercy	4.5	4.5	3.75	4.25	100.00	83.33
Piper	4	4.75	4.75	4.50	118.75	118.75
Reno	4	4	4	4.00	100.00	100.00
Rip	3.25	3.25	3.25	3.25	100.00	100.00
Ruby	3.75	3	2.5	3.08	80.00	66.67
Valentine	3.25	3.25	3	3.17	100.00	92.31
Wizard	4.75	4.5	4.5	4.58	94.74	94.74

Table 14. Behavioral (anxiety) responses of dogs during the open-field test (Continued)

Dog Name	Mean Global Score 1 Pre	Mean Global Score 2 During	Mean Global Score 3 Post	Mean Sessions 1-3	% Change Anxiety (During/pre)	% Change Anxiety (Post/pre)
Day 2						
Ace	4.25	4.25	4.25	4.25	100.00	100.00
Annie	1.5	4	1.5	2.33	266.67	100.00
Baxter	1.5	3	1.5	2.00	200.00	100.00
Bullet	3	4.25	3.25	3.50	141.67	108.33
Dakota	3.25	4	2.5	3.25	123.08	76.92
Honey	2	4.75	3.25	3.33	237.50	162.50
Hunter	4.5	5	3.75	4.42	111.11	83.33
Jimmy	2	4.5	3.25	3.25	225.00	162.50
Macks	3	3.25	3	3.08	108.33	100.00
Mercy	4	4.5	3.75	4.08	112.50	93.75
Piper	4.75	5	4	4.58	105.26	84.21
Reno	3.75	4.25	3.75	3.92	113.33	100.00
Rip	3.25	4	3.25	3.50	123.08	100.00
Ruby	2.25	2.75	2	2.33	122.22	88.89
Valentine	2.75	4.5	3	3.42	163.64	109.09
Wizard	3.75	4.25	3.75	3.92	113.33	100.00

Table 14. Behavioral (anxiety) responses of dogs during the open-field test (Continued)

Dog Name	Mean Global Score 1 Pre	Mean Global Score 2 During	Mean Global Score 3 Post	Mean Sessions 1-3	% Change Anxiety (During/pre)	% Change Anxiety (Post/pre)
Day 3						
Ace	4.25	4.25	4.25	4.25	100.00	100.00
Annie	1.75	1.25	1.25	1.42	71.43	71.43
Baxter	1.5	1	1	1.17	66.67	66.67
Bullet	3.25	3	3	3.08	92.31	92.31
Dakota	4	4	3.25	3.75	100.00	81.25
Honey	3.25	3	3.25	3.17	92.31	100.00
Hunter	4	3.75	3.25	3.67	93.75	81.25
Jimmy	2	2	1	1.67	100.00	50.00
Macks	1.25	1.25	1.25	1.25	100.00	100.00
Mercy	3.75	3.75	3.75	3.75	100.00	100.00
Piper	4.25	4	3.5	3.92	94.12	82.35
Reno	3.75	3.75	3.75	3.75	100.00	100.00
Rip	3.25	1.75	1.5	2.17	53.85	46.15
Ruby	1.75	1.75	1.75	1.75	100.00	100.00
Valentine	1	1	1	1.00	100.00	100.00
Wizard	4.5	4.5	3.75	4.25	100.00	83.33

Table 14. Behavioral (anxiety) responses of dogs during the open-field test (Continued)

Dog Name	Mean Global Score 1 Pre	Mean Global Score 2 During	Mean Global Score 3 Post	Mean Sessions 1-3	% Change Anxiety (During/pre)	% Change Anxiety (Post/pre)
Day 4						
Ace	4.25	4.5	4.25	4.33	105.88	100.00
Annie	1.25	4	1.25	2.17	320.00	100.00
Baxter	1.5	4	2	2.50	266.67	133.33
Bullet	2.75	4.75	4	3.83	172.73	145.45
Dakota	1.75	1.5	1	1.42	85.71	57.14
Honey	1.75	5	3.75	3.50	285.71	214.29
Hunter	3.5	4.75	4	4.08	135.71	114.29
Jimmy	1.25	2	1	1.42	160.00	80.00
Macks	1.25	2.5	1.5	1.75	200.00	120.00
Mercy	4.5	4.5	4	4.33	100.00	88.89
Piper	2.5	4.5	3.5	3.50	180.00	140.00
Reno	3.5	4.5	3.5	3.83	128.57	100.00
Rip	1.5	4.5	1.5	2.50	300.00	100.00
Ruby	1.75	3	2.25	2.33	171.43	128.57
Valentine	1	3	1	1.67	300.00	100.00
Wizard	4	4.5	3.75	4.08	112.50	93.75

Table 14. Behavioral (anxiety) responses of dogs during the open-field test (Continued)

Dog Name	Mean Global Score 1 Pre	Mean Global Score 2 During	Mean Global Score 3 Post	Mean Sessions 1-3	% Change Anxiety (During/pre)	% Change Anxiety (Post/pre)
Day 5						
Ace	4.25	4.25	4.25	4.25	100.00	100.00
Annie	1	1	1	1.00	100.00	100.00
Baxter	1	1	1	1.00	100.00	100.00
Bullet	3.5	3.5	3.5	3.50	100.00	100.00
Dakota	1.5	1	1	1.17	66.67	66.67
Honey	2	2.25	2.25	2.17	112.50	112.50
Hunter	3	3	3	3.00	100.00	100.00
Jimmy	2.25	1.25	1	1.50	55.56	44.44
Macks	1.5	1.25	1.25	1.33	83.33	83.33
Mercy	4.5	4	4	4.17	88.89	88.89
Piper	4.5	3.5	3	3.67	77.78	66.67
Reno	3.5	3.25	2.75	3.17	92.86	78.57
Rip	4	2.5	1.5	2.67	62.50	37.50
Ruby	1.5	1.75	1.75	1.67	116.67	116.67
Valentine	1	1	1	1.00	100.00	100.00
Wizard	3.75	3.75	3.75	3.75	100.00	100.00

Table 15. Non-Worst versus Worst Dogs. Mean difference between global anxiety scores during treatment periods (thunderstorm and gunfire) less pre-treatment periods. Scores for dogs numbered 9-16 defined as “Worst” had scores for mean global (MN Glob) >= 1.000

Obs	Name	Pos	Neg	Global	MN Glob	Worst	ERT Score
1	Mercy	-0.25	0.00	0.25	0.250	0	90
2	Ace	0.00	0.25	0.00	0.125	0	96
3	Dakota	0.75	0.25	0.00	0.250	0	90
4	Hunter	1.00	0.75	1.00	0.875	0	89
5	Macks	1.00	0.50	0.75	0.750	0	84
6	Reno	1.25	0.25	0.75	0.750	0	87
7	Ruby	1.25	0.50	0.75	0.875	0	92
8	Wizard	1.25	0.25	0.50	0.500	0	79
Mean							88.4
9	Piper	0.75	1.50	1.00	1.125	W	54
10	Bullet	1.50	1.75	1.75	1.625	W	91
11	Rip	1.75	2.00	1.75	1.875	W	87
12	Valentine	1.75	2.00	1.75	1.875	W	72
13	Baxter	2.00	2.00	2.00	2.000	W	81
14	Jimmy	2.00	1.25	1.50	1.625	W	88
15	Annie	2.50	2.75	2.50	2.625	W	67
16	Honey	2.75	3.25	2.75	3.000	W	63
Mean							75.4

Table 16. Number of days of pre-training required to reach criteria. Number of errors required to reach criterion during discrimination and reversal training. Object discrimination and reversal criterion: 1 day of 16/20 (80%) or better, followed by 2 days totaling 28/40 or better.

	Pretraining	**Object discrimination**		**Reversal**	
	Total Days	Errors	Total trials	Errors	Total trials
Ace	12	16	80	74	140
Annie	8	41	140	149	300
Baxter	11	34	140	80	180
Bullet	8	12	80	45	140
Dakota	8	17	100	83	200
Honey	17	26	147	105	240
Hunter	10	23	100	60	160
Jimmy	9	33	120	148	280
Macks	8	40	140	67	140
Mercy	12	26	140	91	200
Piper	9	57	219	63	169
Reno	7	24	120	56	160
Rip	9	40	160	133	243
Ruby	8	17	100	60	140
Valentine	8	26	100	134	280
Wizard	10	15	100	92	180

Table 17. Number of errors required to reach criterion on the delayed non match to position (DNMP) task. DNMP criterion: DNMP criterion: First- One session of 11/12 or better; or 2 consecutive sessions of 10/12 or better; or 3 consecutive sessions of 10/12, 9/12, 10/12, in that order. Then- 3 days totaling 26/36 or better (70%).

	DNMP Errors	DNMP total trials
Completed the task	35	108
Ace	35	108
Baxter	60	156
Honey	47	142
Hunter	43	144
Macks	96	276
Mercy	61	168
Ruby	87	252
Valentine	96	240
Wizard	70	204
Did not complete task		
Annie	115	300
Bullet	137	300
Dakota	122	300
Jimmy	138	300
Reno	172	300
Rip	139	300
Did not attempt task		
Piper		

Table 18. Number of errors required to reach criterion on the olfactory discrimination (vanillin : ethanol) task.

	Olfactory- Vanillin	
Dog	Errors	Total trials
Ace	143	340
Annie	60	180
Baxter	145	359
Bullet	95	240
Dakota	140	380
Honey	84	240
Hunter	100	300
Jimmy	101	280
Macks	55	160
Mercy	176	440
Reno	166	380
Rip	64	180
Ruby	141	380
Valentine	151	400
Wizard	62	180

Olfactory criterion 1 day of 16/20 (80%) or better, followed by 2 days totaling 28/40 or better. Non-correction was implemented starting on trial 11.

Table 19. Number of errors required to reach criterion on the AN olfactory discrimination tasks.[a]

	AN : Blank		AN : CV Soil[b]		AN : Amyl acetate		AN:All[c]	
Name	Error	Trials	Error	Trials	Error	Trials	Error	Trial
Ace	10	60	3	40	4	40	17	140
Annie	24	100	4	40	7	40	35	180
Baxter	87	240	13	40	14	60	87	240
Bullet	38	140	11	40	9	60	56	220
Dakota	8	60	7	40	18	80	na	na
Honey	21	100	6	40	4	40	31	180
Hunter	14	80	10	40	4	40	28	160
Jimmy	40	140	5	40	7	40	na	na
Macks	48	200	15	40	8	40	71	280
Mercy	8	60	5	40	8	80	21	180
Piper							na	na
Reno	57	160	4	40	10	40	na	na
Rip	19	100	8	40	3	40	na	na
Ruby	21	80	6	40	6	40	33	160
Valentine	20	80	7	40	6	40	33	160
Wizard	80	220	12	40	8	40	100	300

[a]Olfactory criterion 1 day of 16/20 (80%) or better, followed by 2 days totaling 28/40 or better. Non-correction was implemented starting on trial 11.
[b]Camp Victory (Iraq) soil (compares 5 g of AN to 5 g of CV soil)
[c]Total number of errors and trials during the AN/blank, AN/cv, AN/banana

Table 20. Summary statistics associated with olfactory discrimination training (number of trials required to reach criteria). Number in parentheses gives group size. Mean (± SEM). No statistically significant differences were seen.

Factor	Descriptor	Odor pair Vanillin (S+) : Blank (S-)	Odor pair Ammonium nitrate (S+) : Blank (S-)
Sex	Male	267.4 ± 31.3 (8)	156.7 ± 30.7 (6)
	Female	328.6 ± 36.2 (7)	84.0 ± 7.5 (5)
Coat color	Black	301.9 ± 31.6 (10)	134.3 ± 30.8 (7)
	Yellow	284.0 ± 40.7 (5)	105.0 ± 12.6 (4)
Prior odor training	Yes	296.7 ± 45.4 (6)	100.0 ± 33.7 (4)
	No	295.4 ± 29.4 (9)	137.1 ± 25.2 (7)
Anxiety phenotype	Worse	268.4 ± 32.0 (7)	132.0 ± 28.7 (5)
	Not worse	320.0 ± 35.7 (8)	116.7 ± 29.9 (6)

Table21. Number of training sessions required to reach criteria on the cognitive bias test.

	Pre-training Total Trials
Ace	15
Annie	16
Baxter	33
Bullet	22
Dakota	15
Honey	16
Hunter	26
Jimmy	15
Macks	24
Mercy	43
Piper	17
Reno	18
Rip	33
Ruby	18
Valentine	16
Wizard	24

Table 22. Overall average heart rate, skin temperature, and cumulative activity level in individual dogs for the 20 minute Canine Olfaction Assessment Test (COAT)

Dog	Notes	EKG RR interval (ms) Total Average	EKG Heart Rate bpm Total Average	Temperature °C Total Average	Activity area_sum Total Sum
Ace	16 min	473.11	127.46	40.21	206.37
Annie		655.80	91.63	37.87	30.80
Baxter		836.79	72.36	39.54	15.44
Bullet		687.60	88.04	37.66	114.44
Dakota		546.20	110.27	39.29	26.59
Honey	18 min	641.35	95.81	38.52	55.85
Hunter		694.94	87.45	39.52	35.66
Jimmy		943.23	63.84	39.99	7.70
Macks		598.07	102.15	38.04	153.41
Mercy	noisy, some dropout	601.35	100.95	39.14	31.47
Piper		593.32	106.27	37.02	98.76
Reno		866.72	71.53	39.13	34.98
Rip	noisy	761.39	80.37	37.56	34.69
Ruby		603.59	101.29	39.39	57.53
Valentine		584.08	103.79	38.68	132.71
Wizard	signal drop on one block excluded	501.86	119.74	39.78	70.31
Grand Mean		661.84	95.19	38.83	69.17
Grand STDEV		131.74	17.50	0.96	56.60
Grand SEM		34.02	4.52	0.25	14.61

Table 23. Individual data from dogs in cohort 2 during the C4 priming field experiments.

Dog	Group	Odor Lane(sec)	# Odor lane Falses	Time to detect (sec)	Field-# Falses
Trial 1					
Kody	Blank	32	0	37	0
Annie	Blank	47	0	166	0
Heidi	Blank	47	0	206	0
Charlie	Blank	36	0	**161**	0
Hannah	Blank	50	0	250	0
Ike	Blank	73	1	93	0
Cricket	Pre-scent	12	0	**164**	0
Twiggy	Pre-scent	13	0	60	0
Salty	Pre-scent	7	0	211	0
Brutus	Pre-scent	5	0	127	0
Ally	Pre-scent	8	0	52	0
Harley	Pre-scent	25	0	68	0
Trial 2					
Kody	Blank	86	0	114	0
Annie	Blank	21	0	49	0
Heidi	Blank	51	0	45	0
Charlie	Blank	19	0	77	0
Hannah	Blank	46	0	208	0
Ike	Blank	41	1	126	0
Cricket	Pre-scent	8	0	15	0
Twiggy	Pre-scent	30	0	88	0
Salty	Pre-scent	8	0	58	0
Brutus	Pre-scent	5	0	27	0
Ally	Pre-scent	6	0	108	0
Harley	Pre-scent	12	0	41	0

Table 24. Ability of dogs to cover on 0.25, 2.5, 25, or 250 g of AN (composite positive cover rate is shown).

Dog	Mean cover efficiency (% correct 12 trials)
Twiggy	0.17
Hannah	0.25
Brutus	0.67
Harley	0.67
Kody	0.67
Ally	0.92
Annie	0.92
Cricket	0.92
Charlie	1.00
Heidi	1.00
Ike	1.00
Salty	1.00

Table 25. Summary mean (± SEM) time to detect An or C4 when presented at the surface. Numbers in parentheses provide number of trials for each endpoint. Missing data result from dogs being unavailable for testing that day due to lameness or other medical problems

Dog	25 g AN (n =2)	250 g AN (n = 4-6)	25 g C4 (n =2
Ally	31.1 ± 12.3	53.7 ± 28.5 (5)	18.9 ± 5.5
Annie	59.0 ± 4.4	78.3 ± 13.6 (6)	39.0 ± 24.5
Brutus	35.0 ± 22.8	53.2 ± 12.9 (6)	15.5 ± 5.2
Heidi	31.1 ± 12.6	54.1 ± 13.8 (5)	52.9 ± 36.8
Ike		55.8 ± 14.2 (4)	63.7
Kody	93.5 ± 70.3	28.2 ± 6.9 (5)	12.5
Salty	13.2 ± 4.9	41.6 ± 14.1 (6)	19.0 ± 2.4

Table 26. Frequency of cover behavior seen in dogs exposed to buried AN. In this experiment known quantities of AN were buried 15 com below the surface of a PVC pipe system.

	Amount of "Buried" AN (g)			
Dog	0.25	2.5	25	250
Ally	0.33	0.33	0.67	0.67
Annie	0.67	0.67	0.67	1.00
Brutus	0.50	0.50	1.00	1.00
Charlie	1.00	1.00	1.00	1.00
Cricket	0.00	0.00	1.00	1.00
Heidi	0.00	0.33	0.33	0.67
Ike	0.00	0.33	0.00	0.33
Kody	0.00	0.33	0.67	0.33
Salty	0.33	1.00	1.00	0.67

Table 27. Individual mean values for the time needed to detect AN or C4. Baseline data was collected prior to dogs being placed on Prilosec (1 mg/kg/day, oral) for 1 week. Surface trials. Excludes timed out trials.

			Time to detect (sec)		
Dog	Explosive	Quantity (g)	Control	Baseline	Prilosec
Ally	AN	25		31.1	65.4
Ally	AN	250		94.5	20.7
Ally	C4	25		18.9	16.4
Annie	AN	25		59.0	59.3
Annie	AN	250		78.3	16.3
Annie	C4	25		39.0	16.1
Brutus	AN	25		35.0	28.3
Brutus	AN	250		53.2	20.9
Brutus	C4	25		15.5	15.0
Charlie	AN	25	62.2		
Charlie	AN	250	17.9		
Charlie	C4	25	34.3		
Cricket	AN	25	36.7		
Cricket	AN	250	21.0		
Cricket	C4	25	42.7		
Heidi	AN	25		31.1	35.1
Heidi	AN	250		54.1	13.8
Heidi	C4	25		52.9	17.2
Ike	AN	25			26.4
Ike	AN	250		55.8	14.7
Ike	C4	25		63.7	14.0
Kody	AN	25		93.5	80.4
Kody	AN	250		28.2	56.2
Kody	C4	25		12.5	10.4
Salty	AN	25		13.2	36.4
Salty	AN	250		41.6	16.5
Salty	C4	25		19.0	10.9